Oncogenic Ras Induces Gastrin Gene Expression in Colon Cancer

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Background & Aims: The expression of gastrin, as a tumor growth factor, is significantly increased in some colon cancers compared with the low levels found in normal mucosa. The aim of this study was to elucidate the transcriptional mechanisms of gastrin induction in colon cancer. *Methods:* Gastrin messenger (mRNA) levels and K-*ras* genotype were determined in colon cancer cell lines and surgical specimens. Colon cancer cells were transfected with oncogenic *ras* expression vectors, and transcriptional activity was assayed with gastrin-luciferase reporter genes. *Results:* Colon cancer cell lines and tissues with K-*ras* mutations all had significantly higher gastrin mRNA levels than those that were *ras* wild type. Treatment of several *ras* mutant cell lines with PD98059, an inhibitor of mitogen-activated protein kinase kinase, resulted in a decrease in endogenous gastrin mRNA levels. The effects of *ras* on gastrin expression appeared to be mediated through the gastrin promoter because transfection of oncogenic *ras* and activated *raf* expression vectors both induced gastrin-promoter, luciferase-reporter genes. The inductive effects of oncogenic *ras* could be blocked by the coexpression of dominant negative forms of *raf* and extracellular regulated kinase. *Conclusions:* Oncogenic *ras* induces gastrin gene expression through activation of the Raf-MEK-ERK signal transduction pathway.

Gastrin, a peptide hormone and trophic factor, has long been recognized to stimulate mucosal growth in the upper digestive tract.[1,2] More recent findings suggest that gastrin has proliferative effects in colon cancer as well. Many studies have shown that exogenously administered gastrin stimulated the growth and proliferation of colon cancer cells in culture,[3,4] transplanted colon tumors in mice,[5] and carcinogen-induced tumors in rats.[6] In addition, antagonism of gastrin effects by antigastrin antisera,[7,8] gastrin receptor antagonists,[3,9,10] and antisense gastrin RNA[11] inhibited growth of colon cancer cells in culture and in vivo animal models. There is some controversy about whether gastrin acts on colon cancer by hormonal mechanisms through circulating processed gastrin or through autocrine effects of locally produced, incompletely processed gastrin.[12] Both forms of gastrin have proliferative effects on colonic mucosa,[13] but most colon tumors that produce gastrin express the incompletely processed, glycine-extended form.[14–16]

In the normal colon, gastrin expression is greatest during fetal development and then decreases to a very low level from birth through maturity.[17] Some colon cancers, but not all, have increased levels of gastrin message compared with normal mucosa.[14,18] Although there is not much information about what factors induce gastrin expression, some evidence shows that transforming growth factor α (TGF-α) and epidermal growth factor (EGF) stimulate gastrin transcriptional activity in several colon and pituitary cell lines.[19,20] It is well established that many tumors express autocrine growth factors, but the signaling cascades initiated and transcriptional mechanisms involved are poorly characterized,[21,22] including those responsible for gastrin induction in colon cancer.

The Ras-Raf signal transduction pathway, which is frequently involved in oncogenesis, plays a critical role in regulating genes involved in growth and proliferation.[23–25] Although there is clear evidence that oncogenic Ras can have transforming effects that are Raf independent,[26] it is the Ras-Raf-MEK-ERK pathway that responds to mitogens such as EGF[27] and also regulates the expression of growth factors, such as TGF-α[28] and heparin-binding EGF.[29] Such cases suggest that an autocrine loop can involve Ras signaling and tumor growth factor expression. To determine if there was a correlation between oncogenic activation of Ras and gastrin expression, a panel of human colon cancer cell

Abbreviations used in this paper: EGF, epidermal growth factor; PCR, polymerase chain reaction; RPA, ribonuclease protection assay; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TGF-α, transforming growth factor α.

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lines and several tissue specimens were examined for gastrin expression, *ras* status, and Ras-Raf pathway activity.

Materials and Methods

Cell Lines and Tissues

All cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in the following media: RPMI 1640 (Colo320HSR and Colo205), Dulbecco's modified Eagle medium with high glucose (HT29, Hct116, Caco-2, and LoVo), and Leibovitz's L-15 (SW480), each supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and 2% penicillin/streptomycin (10,000 U/mL). Surgical specimens were snap-frozen on resection and stored at −80°C. Treatment of cells with the MEK inhibitor PD98059 (Calbiochem, La Jolla, CA) was as follows. Cells were plated in complete media (plus fetal bovine serum) at an approximate density of 70% confluency. After 12 hours, the media were changed to serum-free media with 10 or 100 μmol/L PD98059 (0.5% dimethyl sulfoxide) or diluent alone (0.5% dimethyl sulfoxide). Cells were harvested 36 hours later for RNA and protein extraction. Cells appeared to tolerate PD98059 treatment well such that, at the time of harvest, there was no significant difference in cell number or morphology from the control cells (diluent treatment). Thirty-six hours of PD98059 treatment was chosen for maximal effect on gastrin messenger RNA (mRNA) levels because there was no significant change after 12 hours and moderate change after 24 hours.

Ribonuclease Protection Assay

Riboprobes were generated from antisense templates from human gastrin exon 2 (nucleotide 304 digested to nucleotide 210), human gastrin exon 1 and 5′-flanking sequence (nucleotide 109 digested to nucleotide 56), and human cyclophilin (nucleotide 170 digested to nucleotide 103). Riboprobes were prepared by in vitro transcription (Riboprobe System; Promega, Madison, WI) incorporating [α^{32}P]cytidine triphosphate (800 Ci/mmol; Amersham, Arlington Heights, IL). Probes were isolated after urea gel electrophoresis and extraction (shaking in 300 mmol/L sodium acetate, 0.1 mmol/L EDTA, and 0.2% sodium dodecyl sulfate [SDS] at 50°C for 2 hours). Total RNA preparation was performed by a standard guanidine isothiocyanate, phenol extraction method.[30] For the ribonuclease protection assay (RPA),[31] both gastrin (1×10^6 cpm) and cyclophilin (1×10^4 cpm) riboprobes were combined with 150 μg of total RNA in each reaction and hybridized for 16 hours at 57°C (1 mmol/L EDTA; 40 mmol/L piperazine-*N*, *N*′-bis(2-ethanesulfonic acid), pH 6.4; 400 mmol/L NaCl; and 80% formamide). Both riboprobes were added to each assay to control for RNA integrity and quantification. After hybridization, samples were digested with ribonuclease A (20 μg/mL) and ribonuclease T1 (250 U/mL) in 300 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), and 5 mmol/L EDTA at 37°C for 30 minutes. After the digestion was stopped, RNA was precipitated and resolved by denaturing gel electrophoresis. Densitometry was determined from digitally scanned radiographs, and results were normalized to cyclophilin levels. Because cyclophilin message was so abundant, approximately 10-fold less cyclophilin probe was used in Figure 1*A*.

K-*ras* Genotyping

Polymerase chain reaction (PCR)-amplified K-*ras* gene fragments were prepared from each of the cell lines and tissues. Analysis of the K-*ras* genotype at codons 12, 13, and 61 was accomplished by two methods: slot blot Southern hybridization and DNA sequencing of subcloned PCR products. Genomic DNA was extracted from each sample according to standard methods[31] and digested with *Eco*R1. PCR was performed by a standard method incorporating *Taq* polymerase (Perkin-Elmer Corp., Norwalk, CT) with 5% formamide added to the reaction (35 cycles of 80°C for 20 seconds, 48°C for 20 seconds, and 72°C for 30 seconds). Human K-*ras* primers were as follows: codon 12-F, 5′-ATGACTGAATATAAACTTGT; codon 12-R, 5′-CGTCCACAAAATGATTCTG; codon 61-F, 5′-TTCCTACAGGAAGCAAGTAG; and codon 61-R, 5′-CACAAAGAAAGCCCTCCCCA.

Southern Hybridization Analysis

Five hundred nanograms of DNA product from each reaction was denatured and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) for slot blot Southern hybridization. ^{32}P-labeled oligonucleotide probes encompassing codons 12 and 13, containing mutations in each codon respectively for glycine (wild type), cysteine, serine, arginine, valine, aspartate, and alanine, were used for hybridization. After the blots were pretreated with buffer (5× standard saline citrate [SSC], 1% SDS, and 0.2% nonfat dried milk),[31] probe was added and incubated at 65°C for 16 hours. Blots were washed in several changes of 0.2× SSC and 0.1% SDS at 61°C and autoradiographed. Codon 61 PCR products were not analyzed by this method.

DNA Sequence Analysis

PCR products were purified by agarose gel electrophoresis and subcloned into pGEM-T vector (Promega) by standard methods. For each sample, dideoxy sequencing confirmed the genotype identified in the slot blot assays. Codon 61 PCR products from ColoHSR, Colo205, HT29, and Caco-2 were subcloned and sequenced.

Statistical Analysis

Statistical analysis of gastrin RPA and *ras* status used a Mann–Whitney test for the central tendency of two distributions.

ERK Assay

ERK activity was determined by an in vitro immune complex kinase assay[32] with recombinant GST-Elk as the substrate.[33] Protein A sepharose and anti-ERK2 antisera (C14; Santa Cruz Biotechnology, Santa Cruz, CA), which can also

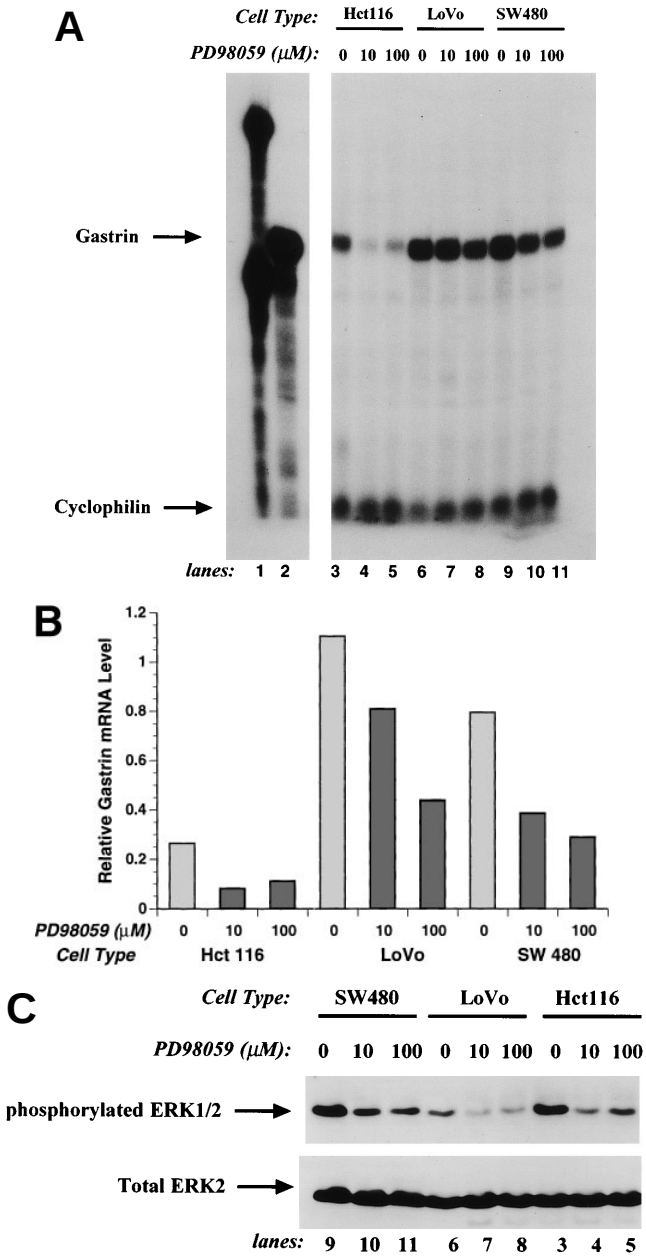

Figure 1. (*A*) RPA of colon cancer cells treated with the MEK inhibitor PD98059. Hct116, LoVo, and SW480 cells were treated for 36 hours with or without inhibitor and subjected to RPA as described in Materials and Methods. Digested gastrin and cyclophilin riboprobes are indicated by the *arrows*. The undigested probes are shown in *lane 1*, and the positive control for gastrin message is shown in *lane 2*. (*B*) Relative gastrin mRNA levels in cells treated with or without PD98059. Densitometry of gastrin and cyclophilin mRNA levels from RPA (*A*) with results normalized to cyclophilin message levels. (*C*) Western blot of phosphorylated and total ERK1,2 from colon cancer cells treated with the MEK inhibitor PD98059. Protein extracts (100 μg each) from cells treated for 36 hours with PD98059 or diluent were resolved by 10% SDS-PAGE and blotted as described in Materials and Methods. Lane numbers refer to the same lanes in *A*. Western blot with antisera and chemiluminescent detection was as described in Materials and Methods.

cross-react with ERK1, were used to immunoprecipitate ERKs from equivalent amounts of whole-cell extracts. After washing the immune complexes, [γ-^{32}P]adenosine triphosphate and recombinant GST-Elk were then added for the kinase reaction. The reaction products were resolved by 10% SDS gel electrophoresis and were autoradiographed.

Western Blot Analysis

ERK1,2 and phosphorylated ERK1,2 levels were determined by Western blot analysis. Equivalent amounts of protein (100 μg) from whole-cell extracts were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene difluoride membranes (Tropifluor; Tropix, Bedford, MA). After blocking nonspecific binding (I-block; Tropix), blots were probed with anti-ERK2 (C14; Santa Cruz) or antiphosphorylated ERK1,2 (Anti-Active MAPK pAb; Promega) antisera. Both antisera are cross-reactive with p44 ERK1 and p42 ERK2. After probing with the primary antisera, blots were washed and probed with anti-rabbit antisera conjugated with alkaline phosphatase. The blots were developed with CDP-Star chemiluminescent reagent (Tropix).

It should be noted that p44 ERK1 was not electrophoretically resolved from p42 ERK2. Hence, ERK1,2 migrated as a single complex. Adjusting the electrophoretic conditions in subsequent confirmatory experiments did achieve resolution of two bands, which when probed with the Anti-Active MAPK antisera showed that both had equivalent degrees of phosphorylation (data not shown).

Transfection and Transcriptional Analysis

Colo HSR cells were plated in six-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ) at approximately 70% confluency in complete media (RPMI 1640 + 10% fetal bovine serum and 2% penicillin/streptomycin). Cells were transfected 12 hours later by a standard $CaPO_4$ technique,[31] and the media were changed to RPMI 1640 supplemented with 0.5% fetal bovine serum. Cells were harvested at 48 hours for luciferase[34] and protein[35] assays. Typically, 1 μg of reporter plasmid and 0.25–0.5 μg of expression plasmid were transfected in each 35-mm well. Construction of gastrin promoter plasmids, 200 GasLuc and 1300 GasLuc, which denote the length of 5′-flanking sequence, has been reported previously.[36] The luciferase reporter plasmids p(Py)2 Luc,[37] pGL Col3 Luc,[38] and pT81 Luc[39] have been described previously. Expression plasmids, including pZip H-ras_{61L} and pZip neo,[40] pZip *raf*CAAX,[26] pZip Rac_{17N}, and pZip Rho_{19N},[41] pVL raf_{301},[42] and pCMV ERK1,2_{KM},[43] have been described previously. Internal transfection standards were not used, but rather luciferase assays were checked for equivalent protein concentrations. Data were derived from the mean of triplicate transfected wells, and transfection experiments were repeated three to four times each.

Results

Gastrin mRNA Levels in Colon Cancer

A sensitive RPA was developed for the detection and quantification of gastrin mRNA levels in colon

cancer cell lines and tissue samples obtained from surgical resection. Antisense riboprobes derived from human gastrin (exon 2) and cyclophilin gene templates were used in each assay. Cyclophilin mRNA, transcribed from a ubiquitous housekeeping gene, served as an internal control for mRNA integrity and reaction conditions. Total RNA samples from seven colon cancer cell lines and two pairs of human tissue samples were used for the RPA shown in Figure 2*A*. Undigested gastrin and cyclophilin riboprobes are present in lane 17. Lanes 1 and 2 contain reactions from a positive gastrin message control and a negative control (transfer RNA only), respectively. Gastrin mRNA was readily detectable in total RNA prepared from SW480, HT29, Hct116, and LoVo cells but was barely discernible in Colo205, ColoHSR, and Caco-2 cells.

Tissue specimens from two colon cancers and their paired normal margins were assayed by gastrin and cyclophilin RPA. Tumor 1 (T1) had significantly elevated gastrin mRNA levels compared with its normal margin tissue (N1). Tumor 2 (T2) had undetectable gastrin message, as did its normal margin (N2). Additional tissue specimens from 9 other colon cancers were assayed by gastrin and cyclophilin RPA as shown in Figure 2*B*. Gastrin mRNA levels were elevated in tumors T3, T4, T6, T8, and T11. Relative amounts of gastrin and cyclophilin mRNAs were determined from both RPAs by densitometry. The cyclophilin mRNA levels were normal-

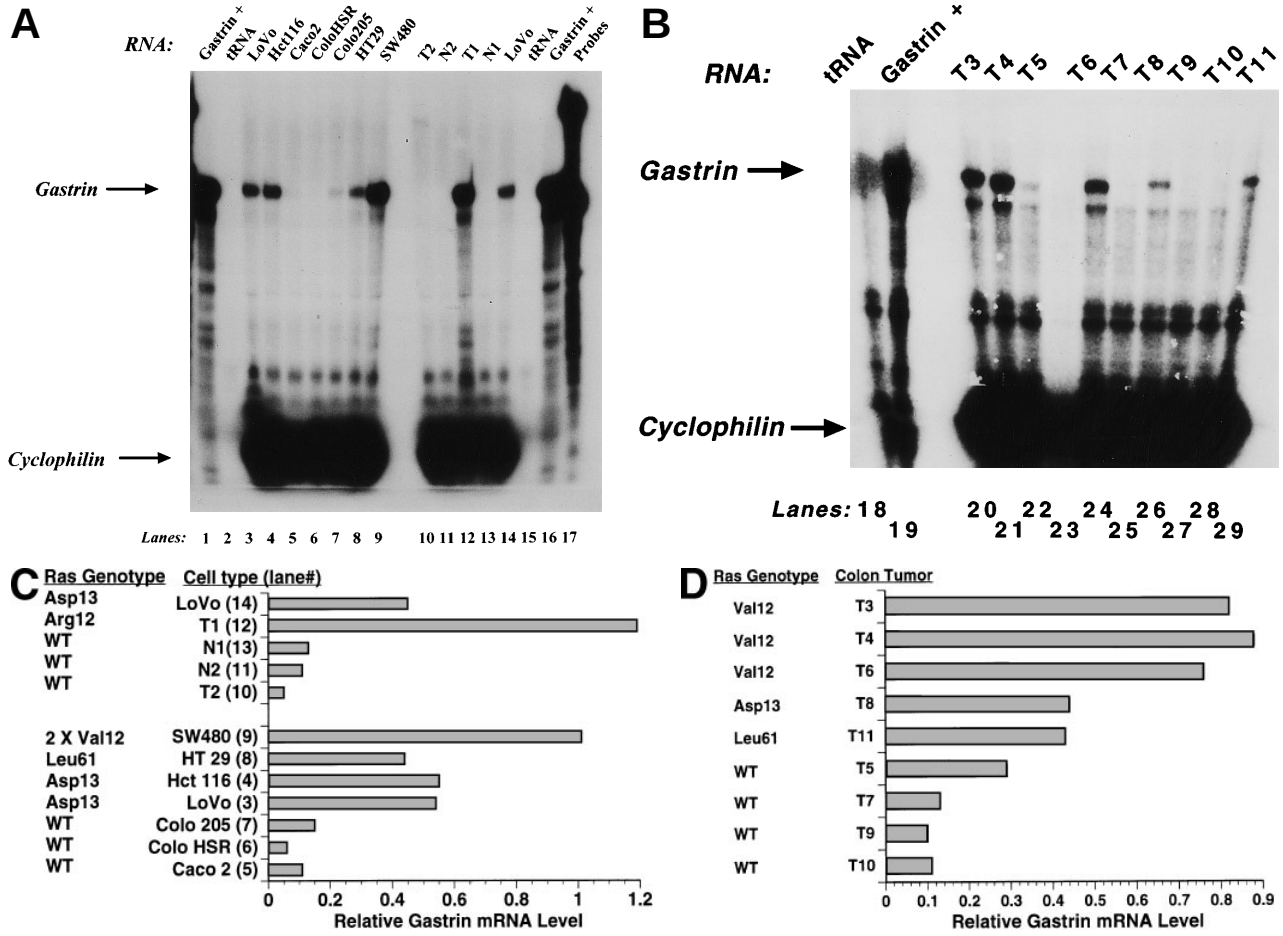

Figure 2. (*A*) RPA for gastrin and cyclophilin message. Total RNA (150 μg each reaction) was hybridized with gastrin and cyclophilin riboprobes and digested as described in Materials and Methods. Digested products are indicated with the *arrows.* Samples were as follows: *lanes 3* and *14,* LoVo; *lane 4,* Hct116; *lane 5,* Caco-2; *lane 6,* ColoHSR; *lane 7,* Colo205; *lane 8,* HT29; *lane 9,* SW480; *lane 10,* tumor 2; *lane 11,* margin 2; *lane 12,* tumor 1; *lane 13,* margin 1; *lanes 2* and *15,* transfer RNA tRNA; *lanes 1* and *16,* human gastrin mouse transgene; and *lane 17,* undigested probes. (*B*) RPA for gastrin and cyclophilin message in colon cancer specimens. Total RNA (150 μg each reaction) was hybridized with gastrin and cyclophilin riboprobes and digested as described in Materials and Methods. Digested products are indicated with the *arrows.* Samples were as follows: *lane 18,* transfer RNA; *lane 19,* human gastrin mouse transgene; *lane 20,* tumor 3; *lane 21,* T4; *lane 22,* T5; *lane 24,* T6; *lane 25,* T7; *lane 26,* T8; *lane 27,* T9; *lane 28,* T10; and *lane 29,* T11. Of note, *lane 18* (transfer RNA control) had a gel artifact of smeared signal from the adjacent lane, and the sample in *lane 23* had degraded RNA. (*C*) Gastrin mRNA levels relative to normalized cyclophilin mRNA. Densitometry of RPA (*A*) with results normalized to cyclophilin message levels. *A* was scanned, and densities were determined for gastrin message levels. A shorter exposure of the radiograph for *A* was used to determine cyclophilin signal densities. K-*ras* genotypes for each sample are listed (see Table 1). WT, wild type. (*D*) Gastrin mRNA levels relative to normalized cyclophilin mRNA of colon cancer specimens. Densitometry of RPA (*B*) with results normalized to cyclophilin message levels. K-*ras* genotypes for each sample are listed (see Table 1).

ized across the samples, and gastrin-relative-to-cyclophilin levels were determined for each sample and plotted as shown in Figure 2*C* and *D*. A >20-fold range in relative gastrin expression was observed between the low-expressing ColoHSR cells and the high-expressing SW480 cells. The results from the surgical samples indicate that, although gastrin expression is typically very low in normal adult colon tissue, it can be significantly elevated in some but not all colon tumors.

Determination of the transcriptional start site was done by hybridization of RNA from several colon cancer cell lines with a riboprobe prepared from a template of human gastrin exon 1 and additional 5′-flanking sequence. This RPA yielded protected fragments identical in length to those obtained from human gastric antral RNA (data not shown), confirming that gastrin mRNA expression in colon cancer occurs from the same transcriptional start site as has been described in other tissues.[44,45]

K-*ras* Genotypes

K-*ras* genotypes were determined from PCR-amplified DNA of each of the cell lines and tissue samples. Mutations in K-*ras* codons 12, 13, and 61 were detected by a combination of allele-specific hybridization and sequencing cloned PCR-amplified regions of the K-*ras* gene. Results of *ras* genotyping are shown in Table 1. Tumors T1, T3, T4, T6, T8, and T11, as well as SW480, HT29, LoVo, and Hct116 cell lines were found to have oncogenic K-*ras* mutations. In contrast, Colo205, Caco-2, and ColoHSR cells; tumors T2, T5, T7, T9, and T10; and both normal colon margin tissues were wild type. Of note, HT29 cells and tumor T11 were found to have an oncogenic mutation in codon 61 (Gln > Leu), which is unusual for K-*ras*. Previous reports of HT29 having wild-type K-*ras* were based on sequencing just the codon 12/13 region.[46,47] All samples with *ras* mutations were heterozygous with a normal allele, with the exception of SW480 which had homozygous *ras* mutations and no detectable normal allele. In each case, the sequence data confirmed the results from the allele-specific oligonucleotide hybridization blots. Comparison of the results from the gastrin RPA and *ras* genotyping shows that the samples possessing *ras* mutations also have the highest gastrin mRNA levels with no overlap between the two groups ($P = 0.002$). Interestingly, of the cell lines with oncogenic *ras* mutations, the only one that was homozygous, SW480, had the highest expression of gastrin. These findings suggest a direct correlation between oncogenic activation of *ras* and induction of gastrin gene expression.

Table 1. K-*ras* Genotypes of Colon Cancer Cell Lines and Surgical Tissue Samples: Confirmation of Southern Oligonucleotide Hybridization by DNA Sequencing

Sample	Codon (mutation)	Genotype
Hct116	13 (GAC)	^{13}Asp
LoVo	13 (GAC)	^{13}Asp
HT29	61 (CTA)	^{61}Leu
SW480	12 (GTT)	^{12}Val homozygous
T1	12 (CGT)	^{12}Arg
T3	12 (GTT)	^{12}Val
T4	12 (GTT)	^{12}Val
T6	12 (GTT)	^{12}Val
T8	13 (GAC)	^{13}Asp
T11	61 (CTA)	^{61}Leu
Caco-2		WT
ColoHSR		WT
Colo205		WT
N1		WT
T2		WT
N2		WT
T5		WT
T7		WT
T9		WT
T10		WT

WT, wild type.

ERK Activity and Phosphorylation

ERK activity was assessed to determine if the cell lines and tissues with oncogenic *ras* mutations have an activated Ras-Raf signal transduction pathway. ERK, which was immunoprecipitated from the cell extracts, was incubated with recombinant GST-Elk protein, a substrate for the kinase enzyme. Phosphorylated GST-Elk was electrophoretically resolved, and the results of the assay are shown in Figure 3*A*. Densitometry of the GST-Elk complex is shown in Figure 3*B*. With the exception of Colo205 (lane 6), *ras* mutant cell lines had greater ERK activity than the wild-type cell lines ColoHSR and Caco-2. Among the tissue samples, only the *ras* mutant tumor 1 (T1) had significantly elevated ERK activity, whereas the other *ras* wild-type tumor sample (T2) was comparable to the normal margin tissue. It is notable that all of the samples appeared to have equivalent amounts of total ERK1,2, as determined by Western blot (Figure 3*C*). These assays suggest that, although total ERK1,2 levels are fairly constant, increased ERK activity tends to occur in those tissues with oncogenic *ras* mutations.

MEK Inhibition and Endogenous Gastrin mRNA Levels

If activation of the Ras-Raf signal transduction pathway is in part responsible for induction of gastrin gene expression, inhibition of the *ras* pathway would likely result in decreased levels of gastrin message. A specific MEK inhibitor, PD98059,[48] was used to treat several colon cancer cell lines, from which total RNA was extracted and assayed for gastrin and cyclophilin message levels. SW480, LoVo, and Hct116 cells, all of which have

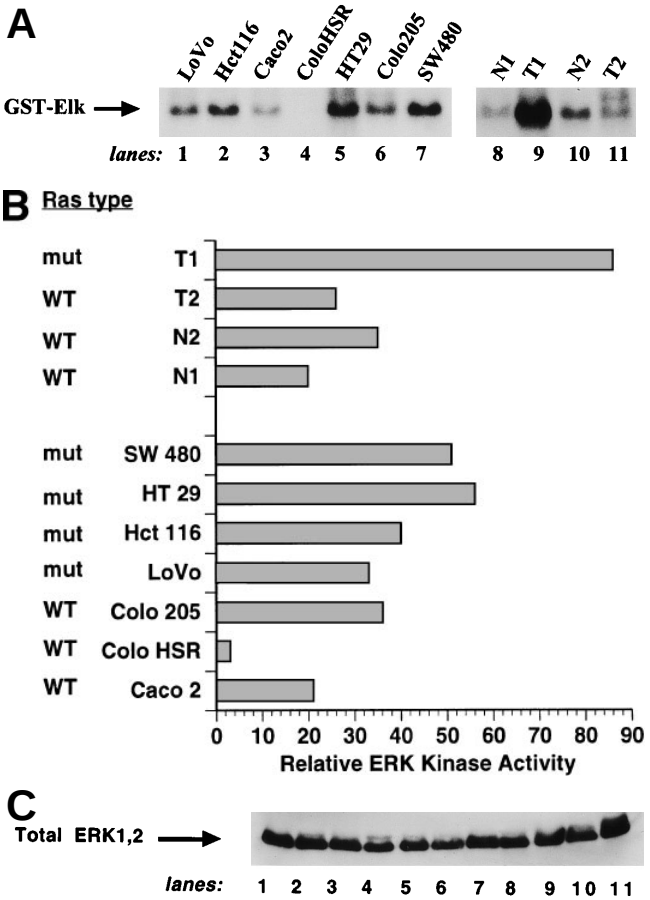

Figure 3. (*A*) ERK kinase activity of colon cancer cells and tissues. Whole-cell and tissue extracts (25 μg protein each) from the same samples shown in Figure 2*A* were used in a kinase assay as described in Materials and Methods. The phosphorylated substrate GST-Elk was electrophoresed, and the autoradiogram was obtained. (*B*) Levels of phosphorylated GST-Elk. Densitometry of ERK kinase assay shown in *A*. (*C*) Levels of ERK1,2 in colon cancer cells and tissues. Western blot of total ERK1,2 from colon cancer cell lines and tissue samples. Whole-cell protein extracts (100 μg each) were resolved by 10% SDS-PAGE, electrotransferred, and probed with anti-ERK2 as described in Materials and Methods. Numbering of lanes corresponds to those in *A*.

oncogenic *ras* mutations, were treated with 0, 10, and 100 μmol/L PD98059. As shown in the RPA (Figure 1*A*), gastrin message levels were significantly lower in treated cells than those controls that were administered vehicle only. There was no significant variation in the cyclophilin message levels. The cyclophilin mRNA levels were normalized across the samples, and gastrin-relative-to-cyclophilin levels were determined for each sample and plotted as shown in Figure 1*B*. These results show that, when controlled for cyclophilin signal intensity, relative gastrin message levels were approximately 60% lower with MEK inhibition. It is noteworthy that gastrin expression in similarly treated ColoHSR cells did not decrease, indicating that the PD98059 had no significant effect on the low basal activity of the Ras-Raf pathway (data not shown).

An indirect measure of kinase activity can be ascertained by probing a Western blot with antisera specific for the phosphorylated forms of ERK. Protein extracts from SW480, LoVo, and Hct116 cells were resolved and blotted by antisera specific for phosphorylated, active ERK1,2 and total ERK1,2 (Figure 1*C*). As shown in Figure 1*C*, Western blot analysis confirmed that PD98059 treatment led to a decrease in activated ERKs without changing the overall ERK protein levels. These results support the conclusion that, in cells with oncogenic *ras* mutations, interference with the Raf-MEK-ERK pathway will down-regulate gastrin mRNA levels, yet not affect levels of the housekeeping gene cyclophilin.

Oncogenic *ras* Activates Gastrin-Promoter, Luciferase-Reporter Genes

Transient transfection experiments with ColoHSR cells, which are *ras* wild type and have low endogenous gastrin expression, were performed to directly examine the transcriptional effects of oncogenic *ras* on the gastrin gene promoter (Figure 4). ColoHSR cells were cotransfected with neomycin or oncogenic *ras* expression vectors and a gastrin-promoter, luciferase-reporter gene containing 200 or 1300 base pairs of gastrin 5′-flanking sequence. Transfection of an H-ras_{61Leu} expression vector stimulated expression of cotransfected gastrin-promoter, luciferase-reporter genes 6–8-fold compared with cells expressing the control neomycin vector. Both lengths of the gastrin promoter seemed to be equally activated,

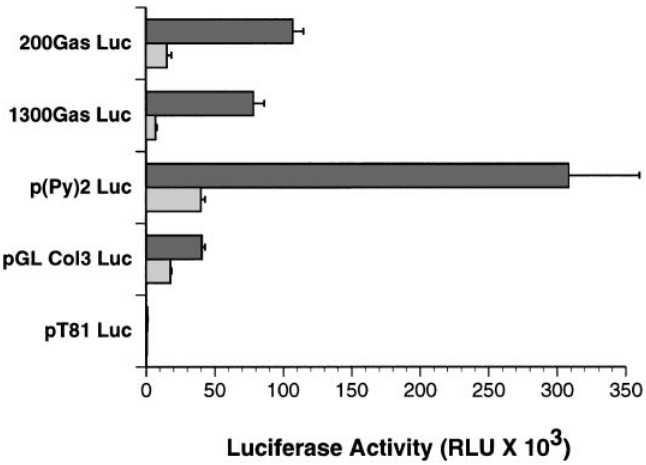

Figure 4. Oncogenic *ras* activation of gastrin-luciferase transcription in ColoHSR cells. Cells were transfected with pZip H-ras_{61L} (■) or pZip neomycin (□) expression vectors (0.25 μg/well) and a reporter plasmid (1 μg) by a $CaPO_4$ transient transfection method as noted. A polyoma viral promoter vector $p(Py)_2$Luc, with tandem *ras* responsive elements served as a positive control, whereas the minimal thymidine kinase promoter vector pT81Luc served as a negative control. A collagen gene promoter vector, pGLCol3Luc, containing 220 base pairs of 5′-flanking sequence, was included for comparison purposes. Luciferase activity was determined at 48 hours. Each data point represents the mean of triplicate determinations (±SEM). RLU, relative light units.

suggesting that the Ras-responsive *cis*-regulatory elements are within the proximal 200 bases of the gastrin 5′-flanking sequence. A polyoma viral promoter vector, p(Py)2Luc, with tandem Ras-responsive elements had approximately 8-fold induction. A collagen gene promoter vector, pGLCol3Luc, had 2-fold induction, whereas the minimal thymidine kinase promoter vector pT81Luc was not induced. These results suggest that oncogenic *ras* can stimulate gastrin transcriptional activity in a gene-specific manner. It is noteworthy that similar results were obtained with transfection of the oncogenic K-ras_{12Val} vector (data not shown), indicating that both forms of oncogenic *ras* can induce gastrin.

Because oncogenic Ras can act along several kinase pathways,[49] the effect of activated Raf on gastrin-luciferase expression was examined next. As shown in Figure 5, transfection of ColoHSR cells with an expression vector for a constitutively active form of Raf, pZIP *raf*CAAX, resulted in 7–10-fold stimulation of gastrin-luciferase expression. Again, the positive control p(Py)2Luc was stimulated, but the collagen and thymidine kinase promoter vectors were not. These results suggest that Ras stimulation of gastrin transcriptional activity is mediated through the Raf-MEK-ERK pathway.

To confirm the specificity of *ras* stimulation of gastrin promoter activity, ColoHSR cells were cotransfected with pZip vectors expressing H-ras_{61Leu}, dominant negative kinases, and the 200 gastrin-luciferase reporter gene. The effects on gastrin-luciferase expression by the cotransfected expression vectors are shown in Figure 6. Coexpression of H-ras_{61Leu} with either dominant negative vectors for *raf* or ERK1,2 resulted in substantial inhibition of the *ras* effect on gastrin-luciferase expression. Dominant negative vectors for *rho* and *rac*, which are collateral *ras*-associated pathways, had no effect on *ras* stimulation of gastrin-luciferase expression. These results and those in the previous figure are consistent with the following: the transcriptional activation of gastrin by oncogenic Ras is mediated principally through the Raf-MEK-ERK signaling pathway.

Because oncogenic Ras apparently increases Raf-MEK-ERK pathway activity and drives gastrin transcription, then one would predict that inhibition of ERKs would have a greater effect on *ras*-transformed cells than those that are *ras* wild type. To test this hypothesis, plasmids for dominant negative ERK kinases or the negative control neomycin were cotransfected with gastrin-luciferase reporter genes into *ras* mutant cell lines LoVo and Hct116 and a *ras* wild-type cell line ColoHSR. Unlike the experiment shown in Figure 6, an oncogenic *ras* expression vector was not cotransfected. As shown in Figure 7, both the *ras* mutant cell lines experienced a >60% decrease in gastrin-luciferase transcriptional activity with the presence of dominant negative ERKs. However, the *ras* wild-type cell line was not significantly affected by the dominant negative ERKs. These results were corroborated by treating Hct116, LoVo, and ColoHSR cells with PD98059 and assaying the resultant gastrin-luciferase transcriptional activity (data not shown). Gastrin-luciferase activity decreased 50% in the *ras* mutant cells but was not affected in the *ras* wild-type ColoHSR. Such findings showing that specific inhibitors of the Ras-Raf pathway down-regulate gastrin-luciferase activity support the conclusion that oncogenic Ras induces gastrin gene expression through transcriptional activation of the gastrin promoter.

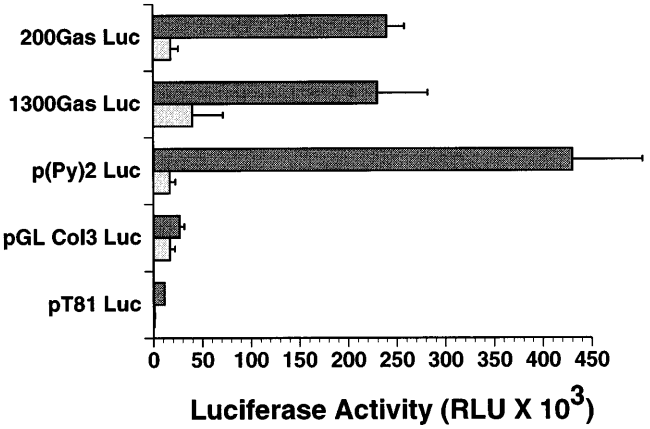

Figure 5. *raf* CAAX activates gastrin-luciferase transcription in ColoHSR cells. Cells were transfected with pZip *raf*CAAX (■) or pZip neomycin (□) expression vectors (0.5 μg/well) and a reporter plasmid (1 μg/well) as described in Materials and Methods. After 48 hours, cells were harvested for luciferase assays, and results of triplicate determinations are shown (±SEM). RLU, relative light units.

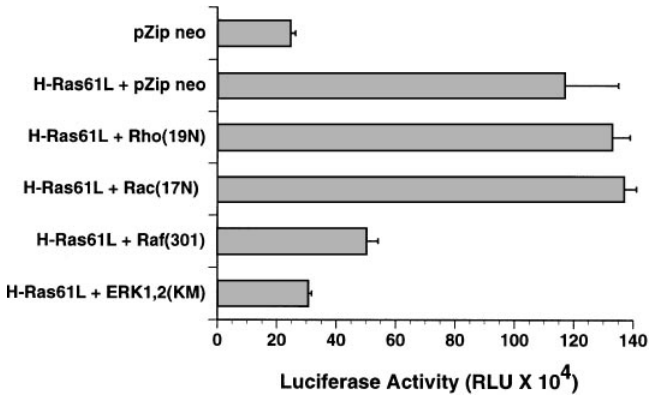

Figure 6. *raf*(301) and ERK1,2(KM) dominant negative kinases partially block H-ras_{61L} activation of gastrin-luciferase transcription in ColoHSR cells. Cells were cotransfected with pZip H-ras_{61L} or pZip neomycin (0.25 μg/well), a dominant negative kinase expression vector (0.5 μg/well), and the 200 Gas Luc reporter plasmid (1 μg/well). Cells were harvested at 48 hours for luciferase assays, and results of triplicate determinations are shown (± SEM). RLU, relative light units.

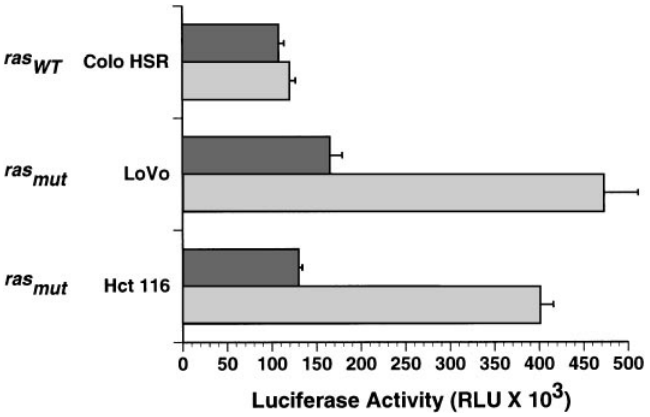

Figure 7. ERK1,2 (KM) dominant negative kinases cause a decrease in gastrin-luciferase transcriptional activity in *ras* mutant cells but not in *ras* wild-type cells. Cells were cotransfected with the 1300 gastrin-luciferase reporter plasmids (1 μg/well) and expression vectors (0.5 μg/well) for dominant negative ERK1,2 (■) or neomycin (□) as described in Materials and Methods. Cells were harvested at 48 hours for luciferase assays, and results of triplicate determinations are shown (± SEM).

Discussion

Several studies have noted that colon cancer tumors often have increased levels of gastrin peptides, particularly incompletely processed forms.[14,15,18] Normal gastrin expression in the colon appears to be much higher during fetal development than after maturity.[17] Hence, the elevated expression of gastrin in colon cancer likely occurs through oncofetal transcriptional mechanisms, which to date have not been well characterized. The results of this study establish that oncogenic activation of the Ras signal transduction pathway induces gastrin gene expression. This evidence is based on the following: (1) a close correlation between the presence of oncogenic K-*ras* mutations and increased gastrin message levels, (2) the down-regulation of endogenous gastrin message levels in cells treated with a specific MEK inhibitor, (3) the stimulation of gastrin transcriptional activity by activated oncogenic *ras* or *raf*, and (4) the ability of specific inhibitors of the Ras-Raf pathway to interfere with gastrin transcriptional activation. This is the first evidence that gastrin is a *ras*-responsive gene and that Ras is acting on the gastrin promoter through a Raf-MEK-ERK pathway.

Oncogenic *ras* is known to induce the expression of other growth factors, such as heparin-binding EGF,[29] vascular endothelial growth factor,[50] and TGF-α.[28] Heparin-binding EGF appears to be an early-response gene responding to Raf induction within several hours.[29] The transcriptional mechanism of Ras-Raf induction of heparin-binding EGF expression involves ERK phosphorylation of an Ets-2 transcription factor and its binding to a composite AP-1/Ets site in the heparin-binding EGF promoter.[51] In contrast, the gastrin gene promoter does not appear to have consensus motifs for AP-1 response elements (Boel et al.[52] and unpublished observations, December, 1997). Of course, it is possible that AP-1 or Ets transcription factors are acting through nonconsensus binding sites.[53] Likewise, a study by Marks et al. indicates that EGF stimulation of gastrin transcriptional activity in pituitary cells depends in part on c-*fos* expression, suggesting a role for AP-1.[54] In addition, they have discovered that in gastric and pituitary cell lines, EGF stimulation of gastrin transcription is mediated through a novel GC-rich proximal *cis*-regulatory gastrin EGF response element.[55] Because the 200–base pair gastrin-luciferase reporter gene is *ras* responsive (Figure 5), it is likely that the *ras*-response elements are located in the proximal gastrin promoter and may include gastrin EGF response element, as well as other known elements. Further studies are indicated to identify *ras*-responsive elements of the gastrin promoter and to determine the transcriptional mechanism of induction.

It is also possible that *ras* induction of gastrin is mediated indirectly through other cytokines or growth factors. For example, *ras* could induce TGF-α expression, which in turn stimulates gastrin expression through an autocrine mechanism. Several studies have shown that *ras* induction of TGF-α expression in rat intestinal epithelial cells leads to an autocrine loop of TGF-α growth stimulation.[28,56] Howell et al. have linked gastrin and TGF-α by showing that gastrin message levels in Hct116 cells fluctuate according to autocrine TGF-α stimulation.[20] They found that when the TGF-α autocrine loop was interrupted by expression of an antisense TGF-α message, gastrin mRNA levels decreased. However, when Baldwin and Zhang analyzed gastrin and TGF-α mRNA levels in seven colon cancer cell lines, they did not find a close correlation between the two factors.[57] Hence, it is possible that stimulation of gastrin gene expression occurs as a secondary event in response to *ras* induction of another growth factor in addition to TGF-α.

We found that, although total ERK levels were equivalent among all the cell lines tested, increased ERK activity generally correlated with the presence of oncogenic *ras* mutations (see Figure 3). However, this association between ERK activity and *ras* status was not as strong as that of oncogenic *ras* and gastrin induction. Nonetheless, these results are consistent with oncogenic *ras* causing constitutive activation of the Raf-MEK-ERK pathway.[33,58] However, Licato et al. recently reported that, although ERK and Jun kinase activities were increased in colon tumors of mice treated with dimethylhydrazine, there was no direct correlation between the ERK activity and *ras* status of the tumor.[59] Although one might expect that ERK activity should always correlate

with *ras* status, it is likely to be more complicated in that other kinases and phosphatases affect the Raf-MEK-ERK pathway.[60–62] Thus, overall ERK activity may be modulated by multiple signal pathways and not limited to the state of Ras activation. It will be important to systematically study larger numbers of tumor samples to determine the degree of correlation between ERK activities and *ras* status.

Several studies have noted that gastrin expression is not uniformly increased in colon cancer tumors.[18,57] A study by Finley et al. noted that, of the colon tumors assayed, more than half of the tumor cells stained for gastrin and 2 of 4 tumors had detectable gastrin mRNA.[18] The results of the current study indicate that gastrin expression is indeed variable but dependent on oncogenic *ras* stimulation. This correlation fits the observations that oncogenic K-*ras* mutations occur in about 50% of the colon tumors.[63] Some studies indicate that tumors with oncogenic K-*ras* mutations are more aggressive and associated with a shorter mean survival time.[64,65] As Singh et al. and Wang et al. have shown, induction of gastrin expression would give the tumors a significant growth advantage.[11,13] Although it remains to be determined whether all colon tumors with oncogenic K-*ras* mutations have increased gastrin expression, it is likely that assessment of both *ras* status and relative gastrin expression will provide useful prognostic information. Such information may be critical for determining which patients with colon cancer will benefit from antigastrin therapy.[11]

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