Regulation of TRAIL Expression by the Phosphatidylinositol 3-Kinase/Akt/GSK-3 Pathway in Human Colon Cancer Cells*

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The intestinal mucosa is a rapidly-renewing tissue characterized by cell proliferation, differentiation, and eventual apoptosis with progression up the vertical gut axis. The inhibition of phosphatidylinositol (PI) 3-kinase by specific chemical inhibitors or overexpression of the lipid phosphatase PTEN enhances enterocyte-like differentiation in human colon cancer cell models of intestinal differentiation. In this report, we examined the role of PI 3-kinase inhibition in the regulation of apoptotic gene expression in human colon cancer cell lines HT29, HCT-116, and Caco-2. Inhibition of PI 3-kinase with the chemical inhibitor wortmannin increased TNF-related apoptosis-inducing ligand (TRAIL; Apo2) mRNA and protein expression. Similarly, overexpression of the tumor suppressor protein PTEN, an antagonist of PI 3-kinase signaling, resulted in the increased expression of TRAIL. Activation of PI 3-kinase by pre-treatment with IGF-1, a gut trophic factor, markedly attenuated the induction of TRAIL by wortmannin. Moreover, overexpression of active Akt, a downstream target of PI 3-kinase, or inhibition of GSK-3, a downstream target of active Akt, completely blocked the induction of TRAIL by wortmannin. Consistent with findings that TRAIL is induced by agents that enhance intestinal cell differentiation, TRAIL expression was specifically localized to the differentiated cells of the colon and small bowel. Adenovirus-mediated overexpression of TRAIL increased DNA fragmentation of HCT-116 cells, demonstrating the functional activity of TRAIL induction. Taken together, our findings demonstrate induction of the TRAIL by inhibition of PI 3-kinase in colon cancer cell lines. These results identify TRAIL, a novel TNF family member, as a downstream target of the PI 3-kinase/Akt/GSK-3 pathway and may have important implications for better understanding the role of the PI 3-kinase pathway in intestinal cell homeostasis.

The epithelium of the mammalian intestine is a dynamic and continuously renewing tissue serving a number of critical physiological functions which, depending upon the location along the cephalocaudal gut axis, include digestion and nutrient absorption, barrier and immune functions, and secretion (1). The intestinal mucosa is characterized by a remarkably efficient and highly regimented progression of proliferation and differentiation with progression of cells up the crypt axis of the colon and the crypt-villus axis of the small bowel (2). Proliferating cells are localized to the lower crypt fractions with differentiated cells localized to the upper half of the colon and the villus fraction of the small bowel. Over a 3–5-day period, the differentiated colonocytes and enterocytes are extruded into the intestinal lumen (3, 4). The cellular mechanisms triggering the differentiation and subsequent extrusion of these epithelial cells are not entirely known.

Phosphatidylinositol 3-kinase (PI 3-kinase); a ubiquitous lipid kinase that is involved in receptor signal transduction through tyrosine kinase receptors, is composed of a regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (5, 6). PI 3-kinase catalyzes the phosphorylation of phosphoinositol 4-phosphate and phosphoinositol 4,5-phosphate at the D3 position (7). It regulates several downstream targets including Akt/protein kinase B (PKB). PI 3-kinase regulates a number of important cellular processes such as cell growth and transformation, membrane ruffling, actin rearrangement, vesicular trafficking, and cell survival. Promotion of cell survival by the activation of PI-3-kinase/Akt occurs by the inhibition of pro-apoptotic signals and the induction of survival signals (7–11), which may contribute to malignant transformation. Conversely, the inhibition of PI-3-kinase/Akt results in cell cycle arrest and differentiation in certain cell types, such as the human colon cancer cell lines HT29 and Caco-2 (12). Glycogen synthase kinase-3 (GSK3) is an Akt substrate shown to be inhibited upon phosphorylation by Akt (13). GSK-3, a component of the Wnt signaling pathway, has been implicated in multiple biological processes by phosphorylation of a broad range of substrates, including several transcription factors such as c-Myc, c-Jun, and c-Myc and the translation factor eIF2B (14, 15). As a downstream target of the PI-3-kinase/Akt pathway, GSK-3 activity suppresses cell proliferation and survival (16, 17). The tumor suppressor gene PTEN (for Phosphatase and tensin homologue deleted on chromosome 10; also called MMAC1 or TEP1) encodes a 403-amino acid phosphatase that antagonizes the activity of PI-3-kinase by dephosphorylating the D3-phosphate group of lipid second messengers, thus serving as a negative regulator of the PI-3-kinase pathway (18). This effect of PTEN inhibits downstream functions mediated by the PI-3-kinase pathway, such as activation of Akt/PKB, cell survival, and cell proliferation (19–21).

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1 The abbreviations used are: PI-3-kinase, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor 1; PTEN, phosphatase and tensin homologue, deleted on chromosome 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; PBS, phosphate-buffered saline; pfu, plaque-forming unit; FITC, fluorescein isothiocyanate.
Members of the tumor necrosis factor (TNF) family interact with their cell surface receptors to directly engage the cellular apoptotic machinery (22, 23). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called Apo-2 ligand), a novel member of the TNF family, is a type II membrane protein identified based on homology to the extracellular domains of TNF and FasL (CD95L) (24, 25). Unlike TNF and FasL, TRAIL is expressed in a variety of cell types and is capable of inducing apoptosis in normal and neoplastic cells (26, 27). In addition, TRAIL blockade results in hyperproliferation of synovial cells and lymphocytes, whereas TRAIL inhibits DNA synthesis in lymphocytes by blocking cell cycle progression (28). Therefore, TRAIL appears to play important roles in cell proliferation and survival; however, little is known regarding the signaling pathways that regulate TRAIL expression.

Recently, we have shown that inhibition of PI 3-kinase, using the chemical inhibitor wortmannin or PTEN overexpression significantly enhances enterocty-like differentiation of the HT29 and Caco-2 human colon cancer cells (12), which display a multipotent phenotype and are well-characterized models of intestinal differentiation (29–34). The purpose of our present study was to identify potential downstream targets of PI 3-kinase inhibition, which may contribute to intestinal cell differentiation and/or apoptosis. Here, we report that the PI 3-kinase signaling pathway negatively regulates TRAIL expression in human colon cancer cell lines. The induction of TRAIL expression by PI 3-kinase inhibition was demonstrated with the PI 3-kinase inhibitor wortmannin or by the constitutive overexpression of Akt or inhibition of GSK-3 completely blocked the induction of TRAIL by wortmannin. Thus, our study identifies the TRAIL gene as a novel downstream target of PI 3-kinase inhibition.

EXPERIMENTAL PROCEDURES

Materials—Wortmannin, actinomycin D, cycloheximide, and lithium chloride were purchased from Sigma (St. Louis, MO) and bis-indolylmaleimide (GF109203x) were from Calbiochem (San Diego, CA). The GSK-3 inhibitors SB-216763 and SB-415286 were gifts from Wataru Ogawa (Kobe University, Kobe, Japan) (37). The adenovirus vector encoding Akt (AxCA-Myr-Akt) was from Wataru Ogawa (Kobe University, Kobe, Japan) (37). Recombinant human TRAIL-R2:Fc was purchased from R&D systems (Minneapolis, MN). Mouse anti-human PTEN monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Akt, anti-phospho-Akt (Ser-473) and rabbit antiphospho-GSK-3α/β (Ser-21/9) antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit anti-β-actin antibody was from Sigma. Mouse antibody against human TRAIL (R2K-2) was a gift from Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan) (35). Recombinant human TRAIL-R2:Fc was purchased from Alexis Corporation (San Diego, CA). Adenovirus vectors encoding β-galactosidase (AdCA-LacZ), control and PTEN (AdCA-PTEN) were from Akira Horii (Tokohu University School of Medicine, Sendai, Japan) (36). The adenovirus vector encoding the myristoylated active form of Akt (AxCA-Myr-Akt) was from Wataru Ogawa (Kobe University School of Medicine, Chuo-ku, Japan) (37). The adenovirus vector-encoding TRAIL (Ad-TRAIL) was purchased from Thomas Griffith (University of Iowa, Iowa City, IA) (38). The human apoptosis DNA template set (haAPO-3c) was from BD Pharmingen (San Diego, CA). γ-32P-ATP (3,000 Ci/mmol) was from Amersham Biosciences. Nitrocellulose filters and x-ray film was purchased from Eastman Kodak (Rochester, NY). The enhanced chemiluminescence (ECL) system for Western immunoblot analysis was from Amersham Biosciences. Tissue culture media and RT-PCR reagents were obtained from Invitrogen. All other reagents were of molecular biology grade and purchased from either Sigma or Amresco (Solon, OH).

Cell Culture—The human colon cancer cell lines HT29 and HCT-116 (ATCC, Manassas, VA) were maintained in McCoy’s 5A supplemented with 1% fetal calf serum (FCS). Caco-2 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Wortmannin was dissolved in dimethyl sulfoxide (DMSO). In all experiments, the effects of wortmannin were compared with cells treated with vehicle (i.e. DMSO at a concentration less than 0.05%). Cells were infected with adenovirus vectors AdCA-PTEN and AxCA-Akt at 10 plaque-forming units (pfu/cell) as described previously (25) and TRAIL at 1000 pfu/cell (38) and incubated for 24 h prior to initiating treatment.

RNA Isolation, RNase Protection, and Northern Blot Analysis—RNA was isolated from cells using Ultraspec RNA reagent according to the manufacturer’s protocol. A 32P-labeled antisense RNA probe was prepared using the Human Apoptosis hAPO-3c Template Set (BD Pharmingen), which measures multiple mRNA species and RNA analyzed as we have previously described (39). Total RNA (40 μg) was run in 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes for Northern blot analysis as described previously (40). Membranes were hybridized to a random-primed 32P-labeled cDNA probe overnight at 43 °C and then washed two times at room temperature with 2× SSC and 0.1% SDS and two times at 45 °C for 15 min with 0.5× SSC and 0.1% SDS. The human TRAIL cDNA probe was synthesized as previously described (41) by RT-PCR using the following primers: 5′-CTTCA-GACTGCTCCGTGACT-3′ and 5′-TTAGCCACAAAAGGGCCC-3′, which is complementary to nucleotides 913−933 of the cDNA sequence. The PCR fragment was sequenced and confirmed to be the correct sequence for human TRAIL. Blots were stripped and reprobed with GAPDH to ensure equal loading. Signals were detected by autoradiography.

Reverse Transcription-PCR (RT-PCR)—A 5-μg aliquot of total RNA was reverse transcribed with Maloney Murine Leukemia Virus (M-MLV) reverse transcriptase, and the resulting cDNA was combined with each primer pair and PCR reagents in a final reaction volume of 50 μl. PCR was carried out for 30 cycles (95 °C melting temperature for 45 s; 60 °C annealing temperature for 45 s; 72 °C extension temperature for 1 min). The following two primers were synthesized: 5′-CTTCA-GACTGCTCCGTGACT-3′, which spans nucleotides 150−169 of the human TRAIL cDNA sequence, and 5′-TTAGCCACAAAAGGGCCC-3′, which is complementary to nucleotides 913−933 of the cDNA sequence. GAPDH was amplified to assess equal loading using the PCR protocol previously described (41).

Protein Preparation and Western Immunoblot—Western immunoblot analyses were performed as described previously (42). Cells were lysed with TNN buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and 25 μg/ml each of aprotinin, leupeptin, and pepstatin A) at 4 °C for 50 min. Lysates were clarified by centrifugation (10,000 g for 30 min at 4 °C) and protein concentrations determined using the method of Bradford (43). Briefly, total protein (100 μg) was resolved on a 10% polyacrylamide gel and transferred to immobilon-P nylon membranes. Filters were incubated overnight at 4 °C in blotting solution (Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20). Akt, phosphorylated Akt, PTEN, β-actin, and phospho-GSK-3α/β antibodies were detected with specific antibodies to these proteins following blotting with a horseradish peroxidase-conjugated secondary antibody and visualized using ECL detection.

Flow Cytometric Analysis—HT29 cells were incubated with wortmannin or vehicle (i.e. DMSO). 5×105 cells in a final volume of 100 μl of PBS were incubated with 1 μg of R2K-2 antibody or isotype control (mouse IgG) for 1 h at 4 °C, washed twice, and resuspended in 100 μl of PBS. For secondary staining, cells were incubated for 45 min at 4 °C in the dark with 1 μg of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. After washing with PBS, the cells were fixed with 1% paraformaldehyde in PBS; specific fluorescence was measured using a FACScan.

Immunofluorescence and Phase Contrast Microscopy—HT29 cells were grown onto sterile glass coverslips in 60-mm dishes and cultured for 16−24 h before treated. Cells were treated with vehicle (DMSO) or Me2SO at 37 °C for 4 h. Cells were washed in PBS, fixed in cold methanol for 5 min, and washed in three changes of PBS followed by a solution of 10% normal goat serum (Sigma). After washing, cells were then incubated with the TRAIL receptor, TRAIL-R2 (also known as DR5), fused with human FC (TRAIL-R2:Fc) (1 μg/ml diluted in PBS with 1.5% normal goat serum) for 60 min. After washing with PBS, cells were
Fig. 1. Wortmannin-induced TRAIL mRNA expression in HT29 cells. A, ribonuclease (RNase) protection assays were performed using RNA from HT29 cells treated with vehicle, Me$_2$SO (DMSO) or wortmannin (250 nM) for 4 h, hybridized with a multiprobe (hAPO-3c; Pharmingen) which assesses a number of apoptotic-related genes. B, Northern blot of total RNA (40 µg) from HT29 cells treated with various concentrations of wortmannin for 4 h and hybridized to the TRAIL cDNA probe. The same membrane was reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as an internal loading control. C, HT29 cells were treated with 250 nM wortmannin for various times and total RNA extracted for TRAIL mRNA detection by Northern blot. D, cells were treated with 250 nM wortmannin and actinomycin D (10 µg/ml) or cycloheximide (9 µM) for 4 h. Total cellular RNA was extracted, and Northern analysis performed using a radiolabeled TRAIL cDNA. The same membrane was stripped and reprobed with a human GAPDH cDNA to indicate relative amounts of hybridizable RNA per lane. E, immunohistochemical analysis of TRAIL protein expression in normal human colon and jejunum. Sections were fixed and stained with a primary mouse monoclonal anti-human TRAIL antibody (Pharmingen). TRAIL is specifically expressed in the differentiated portion of the colon (crypt) and jejunum (villus fraction). Results are representative of the staining pattern noted in the assessment of normal colon and small bowel from 37 separate patient samples.

were incubated with FITC-conjugated goat anti-human FC antibody (2 µg/ml) in PBS with 1.5% normal goat serum for 45 min. After three final washes, the slides were viewed with a fluorescence and phase contrast microscope.

DNA Fragmentation Assay—Cells were plated in 96-well plates 24 h before treatment. After treatment, DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) using a Cell death Detection ELISA Plus kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Immunohistochemical Analysis—Formalin-fixed, paraffin-embedded tissue samples of normal human colon and small bowel were used. Sections (5-µm thick) were fixed to the slide by incubation in a dry oven at 58 °C for 30 min, and then sequentially transferred to xylene (5 min, 2 changes), 100% ethanol (3 min, 2 changes), 95% ethanol (3 min, 2 changes) and rinsed with deionized water. A standard heat-induced epitope retrieval procedure (20 min, 98 °C) was employed by placing slides in commercially available retrieval solution (Target Retrieval Solution, pH 6.0; DAKO, Carpenteria, CA). Slides were allowed to cool at room temperature and rinsed twice with deionized water. Endogenous peroxidase was blocked by placing slides in 3% H$_2$O$_2$/methanol block solution for 10 min, washed with deionized water, and placed in phosphate-buffered saline for 5 min. Slides were incubated at room temperature with primary mouse monoclonal anti-human TRAIL antibody (1:400, BD Pharmingen, cat. 556468) for 30 min. Avidin-biotin peroxidase complex amplification and detection system (LSAB2, DAKO) with diaminobenzidine as chromagen was used. All steps were performed on the automated stainer (DAKO). Negative controls (including no primary antibody or isotype matched mouse IgG) were used in each assessment.

RESULTS

The PI 3-Kinase Inhibitor Wortmannin Induces TRAIL mRNA Expression in HT29 Colon Cancer Cells—Previously, we have shown that inhibition of PI 3-kinase augments the enterocyte-like differentiation of the HT29 and Caco-2 human colon cancer cells (12). These cells undergo differentiation to a small bowel-like phenotype as noted by the induction of brush border enzymes and the presence of microvilli and have been extensively utilized to address mechanistic questions regarding intestinal differentiation (29–34). In this study, we have investigated potential downstream targets of PI 3-kinase inhibition in these intestinal-derived cell lines. HT29 cells were treated for 4 h with the PI 3-kinase inhibitor wortmannin (250 nM), or vehicle control and then the RNA was analyzed by an RNase protection analysis using a multiprobe template (hAPO-3c; BD Pharmingen), which assesses the expression levels of four different genes that contribute to the apoptotic pathway in cells (e.g., TRAIL, Fas, FasL, and TRAIL receptors); L32 and GAPDH are included to ensure equality of loading (Fig. 1A). Wortmannin treatment resulted in the induction of TRAIL gene expression compared with control cells treated with vehicle (i.e., Me$_2$SO).
with no marked change in the expression of the other apoptotic-related genes contained in this probe set.

To confirm the induction of TRAIL by wortmannin, HT29 cells were treated for 4 h with different concentrations of wortmannin (2.5 nM to 1 μM) and Northern blot analysis performed using a human TRAIL cDNA probe (Fig. 1B). Wortmannin treatment increased TRAIL mRNA levels in a dose-dependent fashion with TRAIL induction noted using a dosage of only 2.5 nM. To next assess the time course for TRAIL mRNA induction, HT29 cells were treated with wortmannin (250 nM) for 0.5–8 h (Fig. 1C). Induction of TRAIL mRNA was noted at 2 h after wortmannin treatment with further increases demonstrated at 4 and 8 h.

To determine whether TRAIL induction requires RNA transcription or new protein synthesis, HT29 cells were treated with either actinomycin D (10 μg/ml), which inhibits transcription, or cycloheximide (9 μM), which inhibits protein synthesis, in combination with wortmannin (250 nM) for 4 h, total RNA was extracted and Northern blot analysis was performed (Fig. 1D). Induction of TRAIL by wortmannin was completely blocked by actinomycin D. In contrast, treatment with cycloheximide had no effect on TRAIL induction. Therefore, these findings suggest that TRAIL induction by PI 3-kinase inhibition is regulated at the level of transcription and is not dependent on de novo protein synthesis.

Our findings using the HT29 human colon cancer cell line demonstrate induction of TRAIL expression associated with PI 3-kinase inhibition, which we have previously shown enhances the differentiation of the human colon cancer cell lines HT29 and Caco-2 (12). To determine the location of TRAIL expression in vivo, sections of normal small bowel and colon were obtained from adult patients and analyzed (Fig. 1E). Interestingly, TRAIL expression was specifically localized to the intestinal cells in the differentiated fractions of the intestinal mucosa. That is, intense staining for TRAIL was located in upper crypt portions of the colon (Fig. 1E, left panel) and the villus fraction of the jejunum (Fig. 1E, right panel). Little to no staining of TRAIL was noted in the lower crypt of the colon or crypt cells of the jejunum. Therefore, these findings confirm the induction of TRAIL expression specifically in the more differentiated portions of the intestinal mucosa thus further suggesting the association of TRAIL induction in the HT29 cells with treatments that result in a more differentiated phenotype.

Induction of TRAIL Protein Expression on Wortmannin-treated HT29 Cells—TRAIL is a type II membrane protein; therefore, we next assessed whether PI 3-kinase inhibition affects TRAIL protein expression on HT29 cells using both flow cytometry and immunofluorescence (Fig. 2). HT29 cells were treated with wortmannin (500 nM) for 4 h and then assessed by flow cytometry using an anti-TRAIL antibody (RIK-2) (35). TRAIL expression was detected on the surface of HT29 cells; a shift was observed after wortmannin treatment indicating induction of TRAIL expression (Fig. 2A). Consistent with these results, immunofluorescent staining with TRAIL-R1-FC recombinant protein showed a low level of TRAIL expression on the surface of control cells; increased TRAIL expression was demonstrated on wortmannin-treated HT29 cells (Fig. 2B). Taken together, these results demonstrate that PI 3-kinase inhibition increased both TRAIL mRNA and protein expression in HT29 cells.

Activation of PI 3-Kinase Attenuates TRAIL Induction whereas PTEN Overexpression Induces TRAIL Expression—IGF-1, a trophic factor for intestinal mucosa (44), activates PI 3-kinase with subsequent activation of Akt/ PKB in a number of cell lines (13, 45–47). To determine whether activation of PI 3-kinase can inhibit wortmannin-induced TRAIL expression, HT29 cells were pretreated with IGF-1 (50 ng/ml) for 20 min followed by treatment with wortmannin in the presence of IGF-1 (250 nM) (Fig. 3A). Cells were harvested 4 h later and protein assessed for PI 3-kinase activation through detection of Akt phosphorylation using an antibody specific for phosphorylated (i.e., active) Akt (Fig. 3A, upper panel); IGF-1 stimulated PI 3-kinase as demonstrated by the increased phosphorylation of Akt (lane 3); however, wortmannin treatment attenuated the phosphorylation of Akt (lane 4). As shown in the lower panel (Fig. 3A), wortmannin treatment induced TRAIL expression (lane 2). Treatment with IGF-1 alone had no effect on TRAIL expression (lane 3); however, IGF-1 pretreatment markedly inhibited TRAIL induction by wortmannin (lane 4). These results indicate that TRAIL expression is negatively regulated by PI 3-kinase activation in HT29 cells and are consistent with our findings that PI 3-kinase inhibition induces TRAIL expression.

PTEN is a lipid phosphatase that antagonizes PI 3-kinase activity and has been shown to play a major role in cell cycle arrest and apoptosis (20, 48). We next examined the effect of
PTEN overexpression on TRAIL gene induction. HT29 cells were infected with an adenovirus vector encoding PTEN (AdCA-PTEN) or β-galactosidase (AdCA-LacZ) at an MOI of 10 pfu/cell (Fig. 3B). Infection was carried out for 1 h followed by the replacement of fresh medium and an additional 24 h of incubation. To confirm PTEN overexpression, cells were harvested for protein and Western immunoblot performed demonstrating decreased expression of phosphorylated Akt and PTEN overexpression in HT29 cells infected with the PTEN adenovirus (Fig. 3B, upper panel). Moreover, PTEN overexpression resulted in increased TRAIL expression, as demonstrated by Northern blot, compared with infection of the control β-galactosidase virus (Fig. 3B, lower panel). The increased basal expression of TRAIL noted in the control (β-galactosidase-infected) cells is related to the longer exposure of the film (i.e., ~2 days) compared with overnight exposures shown in the remainder of our studies. Therefore, similar to treatment with wortmannin, overexpression of PTEN increases TRAIL expression in HT29 cells.

Akt Regulates TRAIL Expression Induced by Wortmannin—To further delineate the pathway leading to TRAIL induction with PI 3-kinase inhibition, we next assessed downstream effectors of the PI 3-kinase pathway. First, the role of Akt, a downstream effector of PI 3-kinase, was examined in the regulation of TRAIL expression. HT29 cells were infected with an adenovirus encoding the activated myristoylated form of Akt (AxCA-Myr-Akt) or the adenoviral control vector encoding β-galactosidase at an MOI of 10 pfu/cell. Infection was carried out for 1 h followed by the replacement of fresh medium and an additional 24 h of incubation. Cells were treated with wortmannin or vehicle and protein and RNA extracted for Western and Northern blot analysis, respectively (Fig. 4). Infection with AxCA-Myr-Akt increased phosphorylation of Akt as well as expression of Akt protein (Fig. 4A, lanes 2 and 4); wortmannin treatment had no effect on the increased phosphorylation level of Akt (lane 4). As shown in the lower panel (Fig. 4B), infection of HT29 cells with the AxCA-Myr-Akt adenoviral vector alone had no effect on TRAIL expression as demonstrated by Northern blot (lane 3); however, infection of the AxCA-Myr-Akt vector resulted in a complete inhibition of TRAIL expression induced by wortmannin (lane 4) compared and infection of the control β-galactosidase adenovirus (lane 3), which suggests that signaling through the PI 3-kinase/Akt pathway regulates TRAIL expression induced by wortmannin treatment.

Activation of GSK-3 Is Required in the Induction of TRAIL by Wortmannin—GSK-3 is inactivated when it is phosphorylated by Akt (49). Hence, it would be predicted that activation of Akt by PI 3-kinase would be associated with inhibition of GSK-3 and, conversely, wortmannin treatment would dephosphorylate and activate GSK-3 by inhibition of PI 3-kinase. Therefore, we examined the effects of various chemical inhibitors of GSK-3 on the induction of TRAIL mRNA expression by wortmannin (Fig. 5). HT29 cells were pretreated with lithium chloride (LiCl), a potent GSK-3 inhibitor, at various concentrations for 30 min followed by treatment with wortmannin in the presence of LiCl as indicated in Fig. 5A. LiCl dose-dependently inhibited wortmannin-induced TRAIL mRNA expression. In control experiments, we found that sodium chloride (NaCl), a monovalent ion control, had no effect on TRAIL expression. We tested two other chemical inhibitors of GSK-3, Ro-318220 (IC50 6.8 nM) and GF109203x (IC50 360 nM) (50). As shown in Fig. 5B, both of these compounds inhibited the induction of TRAIL by wortmannin with Ro-318220 (2 μM) completely blocking TRAIL induction and GF109203x (2 μM) significantly attenuating TRAIL mRNA expression, which correlates with their established efficacy as GSK-3 inhibitors (50).

Although useful as GSK-3 inhibitors, LiCl, Ro-318220, and GF109203x are not entirely selective for GSK-3 and have several additional targets, such as protein kinase C (50, 51). Therefore, we tested two recently described specific GSK-3 inhibitors for their ability to inhibit TRAIL induction. SB-216763 and SB-415286 are structurally distinct maleimides.
that are potent inhibitors of GSK-3β in an ATP competitive manner, and the specificity of these antagonists has been established in assays against 25 different kinases (52, 53). Both compounds completely blocked induction of TRAIL by wortmannin (Fig. 5C). To assay the effect of wortmannin on GSK-3 activity, the phosphorylation level of GSK-3 was determined. Cells were treated with Me2SO control or wortmannin (250 nM) for 4 h and whole cell protein extracted and Western blot performed using an antiphospho-GSK-3β antibody (Fig. 5D). Treatment with wortmannin significantly dephosphorylated GSK-3β, indicating an activation of GSK-3β activity. These data indicate that GSK-3 activity is required for TRAIL induction by wortmannin. Taken together, our results indicate that wortmannin-induced TRAIL expression is through the inhibition of PI 3-kinase/Akt and the subsequent activation of GSK-3.

Inhibition of PI 3-Kinase Increases TRAIL Expression in the HCT-116 and Caco-2 Colon Cancer Cell Lines—We have shown that inhibition of PI 3-kinase by either wortmannin or PTEN overexpression induces TRAIL expression in the HT29 colon cancer cell line. To determine whether this induction occurs in other colon cancer cells, we analyzed TRAIL expression in two other human colon cancer cell lines, HCT-116 and Caco-2. Cells were incubated in the presence of wortmannin (250 nM) or vehicle control for 4 h; total RNA was extracted and RT-PCR performed. Wortmannin induced TRAIL mRNA expression in both of these cell lines compared with control (Fig. 6A). In addition, overexpression of PTEN by infection with the adenoviral PTEN vector increased TRAIL expression compared with the control β-galactosidase vector (Fig. 6B). To confirm PTEN overexpression, cell extracts were analyzed by Western immunoblot demonstrating decreased phosphorylated Akt expression and PTEN overexpression in HCT-116 and Caco-2 cells infected with the PTEN adenovirus (Fig. 6C). Thus, our results demonstrate TRAIL induction by the PI 3-kinase/PTEN signaling pathway in the intestinal-derived HT29, HCT-116, and Caco-2 human colon cancer cells.

Functional Activity of Induced TRAIL Expression in Colon Cancer Cells—Exogenous TRAIL treatment induces apoptosis in the sensitive HCT-116 cells, whereas HT29 and Caco-2 cells are resistant (54, 55). To further assess the functional effects of increased TRAIL expression in these colon cancer cells, we...
infected the cells with an adenovirus encoding TRAIL (Ad-TRAIL) or the control virus (Ad-β-gal; control) at an MOI of 1,000 for 4 h. TRAIL mRNA expression was assayed by RPA 24 h later. As expected, infection with Ad-TRAIL increased TRAIL expression in all three cell lines (Fig. 7A). To examine the functional consequences of Ad-TRAIL infection, the tumor cells were infected with the adenoviral constructs for 4 h, cultured for an additional 24 h, and cell death measured by analyzing DNA fragmentation (Fig. 7B). Infection of the TRAIL-sensitive HCT-116 cells with Ad-TRAIL resulted in a significant increase in DNA fragmentation; in contrast, no increase in cell death was noted in the TRAIL-resistant Caco-2 and HT29 cells. The caspase inhibitor Z-VAD-fmk completely inhibited HCT-116 cell death induced by infection with Ad-TRAIL, thus confirming the importance of caspase activation in the death of Ad-TRAIL-infected HCT-116 cells. Therefore, consistent with previous studies assessing treatment of these cells with exogenous TRAIL (45, 55), an increase in endogenous TRAIL by overexpression produced a similar cell death pattern.

**DISCUSSION**

Previously, we have shown that the inhibition of PI 3-kinase enhances enterocyte-like differentiation of the HT29 and Caco-2 human colon cancer cells suggesting a role for PI 3-kinase inhibition in intestinal cell differentiation (12). In our current study, we show that the PI 3-kinase signaling pathway negatively regulates expression of TRAIL, a member of the TNF superfamily, in human colon cancers. Induction of TRAIL expression was demonstrated by PI 3-kinase inhibition using the chemical inhibitor wortmannin at dosages consistent with a specific inhibitory effect (11). Conversely, activation of PI 3-kinase by IGF-1 markedly attenuated wortmannin-mediated TRAIL induction while overexpression of PTEN induced TRAIL expression, thus identifying the TRAIL gene as a downstream target of PI 3-kinase inhibition in human colon cancer cells. This induction was specific for TRAIL in these cells since the expression of FasL, another member of the TNF family, which, similar to TRAIL, induces apoptosis through a caspase-dependent pathway (56), was not affected by wortmannin treatment. Similar to our findings showing induction of TRAIL by PI 3-kinase inhibition, Suhara et al. (56) recently reported that inhibition of PI 3-kinase up-regulated FasL expression in vascular smooth muscle cells. Taken together with our current findings, these studies indicate that the inhibition of PI 3-kinase can result in the induction of apoptotic-related proteins, such as TRAIL or FasL in a cell type-dependent fashion. PI 3-kinase activation can promote cell survival by the activation of downstream effector proteins (7–11); therefore, the finding that PI 3-kinase inhibition results in the induction of genes, which contribute to cell death is reasonable and further supports the notion that PI 3-kinase plays a major role in the regulation of proliferative signals in certain cells.

The PTEN tumor suppressor gene encodes a multifunctional phosphatase that plays a critical physiologic role in inhibiting the PI 3-kinase pathway and downstream functions of PI 3-kinase such as cell survival and proliferation (57–59). Moreover, current genetic data suggest that PTEN function is required for normal development and differentiation (12, 57). In this regard, our preliminary results suggest that PTEN expression is
localized to the more differentiated cells of the colonic epithelium. Overexpression of PTEN in HT29 and Caco-2 cells significantly augments the induction of brush border enzyme activity, which further identifies a role for PTEN in the process of intestinal cell differentiation (12). Similar to our findings demonstrating induction of TRAIL by wortmannin, we show that TRAIL induction in the colon cancer cell lines occurs by PTEN overexpression thus further confirming a role for PI 3-kinase inhibition in the induction of TRAIL expression. Recently, Matsushima-Nishiu et al. (48) analyzed genes that were up-regulated with overexpression of PTEN in endometrial cancer cell lines by cDNA microarray. Notably, induction of members of the TNF-receptor family and TNF-associated genes was identified. Although TRAIL was not specifically analyzed and not all of the gene changes identified by gene array were confirmed by RT-PCR or conventional hybridization methods, this study suggests that PTEN-mediated up-regulation of TNF superfamily members may represent an important cellular function of PTEN. This up-regulation of TNF-associated genes may contribute to the subsequent apoptosis noted in some cells by PTEN overexpression. Furthermore, the regulation of the TNF-associated genes by PI 3-kinase inhibition may represent an important cellular mechanism for regulating proliferation and cell death.

TRAIL is expressed in a number of tissues and displays potent apoptotic activity against selected targets including a variety of cancers (22, 23, 26, 27). In addition to its well-described effects on cell death, TRAIL can inhibit cell cycle progression whereas blockade of TRAIL results in hyperproliferation in autoreactive lymphocytes, which are resistant to TRAIL-induced apoptosis (28, 60) thus further implicating a physiologic role for TRAIL in certain cells. In our present study, we demonstrate that overexpression of TRAIL in the HCT-116 cell line, which is sensitive to exogenous TRAIL treatment (54), results in enhanced DNA fragmentation and cell death, which was blocked by caspase inhibition. These results, in combination with our findings of a spatial-specific pattern of TRAIL expression along the vertical axis of the small bowel and colon, strongly suggests a role for TRAIL in intestinal homeostasis. Consistent with our findings, Strater et al. (55) recently demonstrated a similar pattern of TRAIL expression localized predominantly to the luminal surface epithelium of the colon. In addition, the TRAIL receptor (TRAIL-R2/DR-5) was coexpressed with TRAIL, and it was postulated that TRAIL may play a role in the early elimination of virus-infected epithelial cells in the normal gut. Collectively, our present study as well as the findings by Strater et al. (55) identify TRAIL as a potentially important protein for intestinal cell homeostasis. The precise role for TRAIL in the intestine remains to be fully delineated.

The PI 3-kinase signaling pathway has been implicated in the growth and apoptosis of various cell types (7–11). Activation of PI 3-kinase by growth factors, such as IGF-1, results in the local accumulation of PtdIns-3,4,5-P3 at the plasma membrane. Newly synthesized PtdIns-3,4,5-P3 recruits Akt/PKB to the plasma membrane where the combination of lipid binding and phosphorylation by PDK-1 serves to further phosphorylate the downstream substrates, such as GSK-3 (61, 62). We found that constitutively active Akt completely prevented the TRAIL induction observed with PI 3-kinase blockade, implicating the regulation of TRAIL expression by PI 3-kinase passes through Akt. Phosphorylation and activation of Akt contribute to increased cell survival and malignant transformation acting through downstream effector proteins such as the pro-apoptotic BAD protein (63), caspase-9 (64), and the transcription factors CREB (65), NF-kB (16), and Forkhead (17). Furthermore, Akt is involved in the phosphorylation and inactivation of GSK-3. Activation of GSK-3 induces apoptosis while inhibition has been shown to reduce apoptosis and enhance cell survival (66, 67), implicating GSK-3 as a central element in the PI 3-kinase/Akt survival pathway. Overexpression of Akt or inhibition of GSK-3 completely blocked TRAIL induction by wortmannin, thus demonstrating regulation of TRAIL expression through the PI 3-kinase/Akt/GSK-3 pathway.

Our current study demonstrates a role for PI 3-kinase inhibition in the induction of TRAIL expression in human colon cancer cells. However, in contrast to our findings, Musgrave et al. (68) reported that anti-CD3-induced TRAIL expression in T-cells was blocked by the PI 3-kinase inhibitors, wortmannin and LY294002. These conflicting results may be explained by differences in cell type and the fact that common signaling mechanisms may be interpreted differently depending on the cellular context. This is further supported by the fact that PI 3-kinase activation may result in cell survival or differentiation depending upon the particular cell type. For example, the inhibition of PI 3-kinase enhances enteroctye-like differentiation of colon cancer cells and induces B16 melanoma cell differentiation (12, 69). However, in contrast, PI 3-kinase inhibition blocks myogenic and adipocyte differentiation (70, 71). In conclusion, our results indicate that TRAIL expression is regulated in human colon cancer cells by the PI 3-kinase/Akt/GSK-3 signaling pathway. Importantly, these findings add the TNF-related TRAIL gene to the growing list of apoptosis-related proteins regulated by the PI 3-kinase pathway. Moreover, our results provide a better understanding of the potential role of the PI 3-kinase pathway in intestinal cell homeostasis.

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