Colon carcinoma cells harboring *PIK3CA* mutations display resistance to growth factor deprivation induced apoptosis

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Abstract

PIK3CA, **encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), is mutated in a variety of human cancers. We screened the colon cancer cell lines previously established in our laboratory for *PIK3CA* mutations and found that four of them harbored gain of function mutations. We have now compared a panel of mutant and wild-type cell lines for cell proliferation and survival in response to stress. There was little difference in PI3K activity between mutant *PIK3CA*-bearing cells (mutant cells) and wild-type *PIK3CA*-bearing cells (wild-type cells) under optimal growth conditions. However, the mutant cells showed constitutive PI3K activity during growth factor deprivation stress (GFDS), whereas PI3K activity decayed rapidly in the wild-type cells. Importantly, constitutively active PI3K rendered the mutant cells resistant to GFDS-induced apoptosis relative to the wild-type cells, indicating a biological advantage under stress conditions that is imparted by the mutant enzymes. Compared with the wild-type cells, the mutant cells were hypersensitive to the apoptosis induced by the PI3K inhibitor LY294002. In addition, *PIK3CA* small interfering RNA significantly decreased DNA synthesis and/or induced apoptosis in the mutant cells but not in the wild-type cells. Furthermore, ecotopic expression of a mutant *PIK3CA* in a nontumorigenic *PIK3CA* wild-type cell line resulted in resistance to GFDS-induced apoptosis, whereas transfection of wild-type *PIK3CA* or empty vector had little effect. Taken together, our studies show that mutant *PIK3CA* increases the capacity for proliferation and survival under environmental stresses, such as GFDS while also imparting greater dependency on the PI3K pathway for proliferation and survival. [Mol Cancer Ther 2007;6(3):1143–50]**

Received 9/8/06; revised 12/21/06; accepted 1/31/07.

Grant support: NIH grants CA 34432, CA54807, and CA 16056.

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doi:10.1158/1535-7163.MCT-06-0555

Introduction

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that catalyzes the transfer of the γ-phosphate from ATP to the D3 position of the phosphatidylinositol, generating phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (1). Class I PI3Ks are heterodimers consisting of two subunits: an adaptor/regulatory subunit and a catalytic subunit (p110). The regulatory subunit is a 50-kDa or 85-kDa protein that is tightly associated with the p110 catalytic subunit. PI3Ks are activated through receptor tyrosine kinases or through G protein–coupled receptors. Class I_A PI3Ks are very diverse in mammals; they have three catalytic p110 isoforms (p110α, β, and δ), each encoded by a separate gene, and seven adaptor proteins generated by expression and alternative splicing of three different genes (p85α, β, and p55γ; ref. 2).

PI3K activation has been shown to have important roles in sustaining processes important to malignancy, including cell proliferation, adhesion, survival, and motility. There are several downstream effectors of PI3K, including PDK1, Rac, p70S6K, certain isoforms of protein kinase C, and AKT, which is most relevant to cell survival (1). Phosphorylation of phosphatidylinositol-4,5-bisphosphate by PI3K generates phosphatidylinositol 3,4,5-trisphosphate, which then recruits PDK1 to the cell membrane. PDK1 subsequently phosphorylates and activates AKT. Activated AKT can then phosphorylate a variety of substrates, such as Bad, FKHR, caspase-9, and GSK3, that participate in a variety of cellular processes, including cell survival and proliferation. Phosphorylation of Bad and caspase-9 by AKT inhibits the ability of these proteins to induce apoptosis. Therefore, the PI3K pathway has been shown to inhibit apoptotic processes and has been linked to inappropriate cell survival by malignant cells in response to stress (3, 4).

Overexpression of AKT has an antiapoptotic effect in many cell types, resulting in a delay of cell death during stress. AKT was found to be amplified in human breast (5, 6), ovarian (7), pancreatic (8), and prostate cancer (9), suggesting the specific involvement of AKT in the onset and/or propagation of cancer. In addition, the genomic locus encoding the p110α catalytic subunit *PIK3CA* was

found to be amplified in a high percentage of various cancers. *PIK3CA* gene amplification has been shown to be associated with the progression of several types of tumors to an invasive phenotype (10–12). Recent studies have shown that *PIK3CA* is mutated in approximately one-third of 234 colorectal cancers but only in 2 of 76 adenomas (13). It is also mutated at a high frequency in other cancers, including breast, ovarian, and hepatocellular carcinomas (14–16), making it one of the most common mutations described to date in human cancers. Expression of one of the *PIK3CA* mutants in NIH3T3 cells conferred significantly more lipid kinase activity compared with the wild-type protein, suggesting the gain-of-function nature of the mutations (13). In addition, some of the most frequent mutants of *PIK3CA* showed transforming activity with high efficiency in chicken embryo fibroblasts, as well as in NIH3T3 cells (17, 18). Analogy to gain-of-function epidermal growth factor receptor (EGFR) mutants in lung cancers suggests that these PIK3CA mutations may be associated with ''oncogenic addiction'' and are, therefore, hypersensitive to PI3K inhibition. Thus, mutant PI3K may be a promising target for PI3K small molecule inhibitors. Exploration of the roles of mutant PI3K in cancer growth, survival, and metastasis will help unveil the mechanisms of malignant progression and facilitate development of drugs for cancer treatment.

This study compares a panel of unmodified *PIK3CA* mutant and wild-type colon cell lines for proliferation and survival under stress condition. We found that mutant *PIK3CA*-bearing cells were more resistant to growth factor deprivation stress (GFDS)–induced apoptosis than the wild-type *PIK3CA*-bearing cells, probably due to the constitutively active PI3K and its downstream effectors. In addition, the mutant cells were hypersensitive to a potent PI3K inhibitor, LY294002, as reflected by significantly increased apoptosis compared with the wild-type cells. *PIK3CA* small interfering RNA dramatically decreased DNA synthesis and induced apoptosis in the mutant cells but not in the wild-type cells. Ectopic expression of mutant, but not wild-type PI3K in the wild-type cells, led to resistance to GFDS-induced apoptosis. Our results suggest that mutant *PIK3CA* confers increased growth and/or survival capacity to colon cancer cells and that the mutant cells possess an increased requirement for the PI3K signaling pathway. Therefore, the constitutively active PI3K/AKT signaling in the mutant PI3K cells might provide an additional therapeutic target for colon cancer treatment.

Our study is complementary to a recent study by Samuels et al., in which they knocked out the wild-type or mutant *PIK3CA* allele in HCT116 or DLD1 colon cancer cells and showed that haploid *PIK3CA* cells, bearing only gain-of-function mutant *PIK3CA*, had reduced cellular dependence on growth factors and were more sensitive to PI3K inhibition by LY294002 relative to haploid wild-type *PIK3CA*-bearing cells (19). Consequently, some of the results observed in the Samuels study could have reflected haploid insufficiency. In contrast, our study was done with intact diploid cells, thus reducing the potential effects resulting from *PIK3CA* haplo-insufficient cells. It has been shown that expression of mutant PI3K enhances lipid kinase activity and induces constitutive activation of AKT signaling and oncogenic transformation in chicken embryo fibroblasts, as well as in NIH3T3 cells (17, 18). Because environmental restriction on growth seems to be common in solid tumors (20), the ability of malignant cells to withstand these stresses is considered a key factor in tumor development and progression. Our finding that the differential cell survival characteristics between the mutant and wild-type cells under stress conditions, in which the wild-type PI3K activity is more severely down-regulated, provides an explanation for the irrelevance of the wild-type PI3K activity in terms of the advantage provided by the mutant enzyme. We also found that ectopic expression of the mutant PI3K in the wild-type PI3K-bearing colon cancer cells led to increased resistance to GFDS-induced apoptosis. This result indicates that gain-of-function mutant *PIK3CA* may contribute to the ability to withstand the environmental stresses that are important in tumor development and/or progression.

Materials and Methods

Cell Culture and Reagents

Human colon carcinoma cells used in this study were established *in vitro* from primary tumors, as described previously (21). HCT116, FET, GEO, CBS, and RCA were cultured in SM medium [McCoy's 5A serum-free medium (Sigma, St. Louis, MO) with pyruvate, vitamins, amino acids, and antibiotics] supplemented with 10 ng/mL epidermal growth factor, 20 μg/mL insulin, and 4 μg/mL transferrin, as described previously (22). RKO and TENN were cultured in SM medium supplemented with 10% fetal bovine serum. Vaco481 were cultured in MEM basal medium (MEM medium with glutamine and amino acids) supplemented with 100 μg/mL insulin, 20 μg/mL transferrin, 8.6 ng/mL selenium, and 2% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. When the cells were under GFDS, they were cultured in SM medium or MEM basal medium without growth factor or serum supplements for the indicated time periods without medium change in between.

Antibodies for caspase-3, caspase-9, poly (ADP-ribose) polymerase (PARP), Akt, phosphorylated (p) Akt (Ser^{473}), PDK1, pPDK1 (Ser^{241}), pBAD (Ser^{136}), and pFKHR (Thr^{24}) were obtained from Cell Signaling Technology (Beverly, MA). Actin antibody was from Sigma. LY294002 was purchased from Calbiochem (La Jolla, CA).

PCR, Sequencing, and Mutational Analysis

Human colon carcinoma cell lines were screened for *PIK3CA* mutations by direct sequencing of all 20 exons and splice junction regions. Primer pairs for each exon were designed using the Primer3 program and positioned at least 25 bp upstream of the 3′ and 5′ splice junction site (details available on request). PCR amplification of the individual exons was done through 35 cycles using standard procedures. PCR products were sequenced in both directions using an ABI Prism 3100.

PI3K Assay

The cells were washed with PBS and lysed in lysis buffer [137 mmol/L NaCl, 20 mmol/L Tris (pH 7.4), 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1% NP40, 100 μmol/L Na_3VO_4, and 1 mmol/L phenylmethylsulfonyl fluoride]. Protein concentrations were determined by bicinchoninic acid, a protein assay reagent (Pierce, Rockford, IL). Lysates (400 μg of protein) were incubated with p85 antibody (Upstate, Charlottesville, VA) at 4°C overnight, followed by further incubation with protein A-agarose for 2 h. Immune complexes were washed twice with each wash buffer: PI3K wash 1, PBS 1% NP40/Na_3VO_4; PI3K wash 2, 100 mmol/L Tris (pH 7.4) and 5 mmol/L LiCl/Na_3VO_4; PI3K wash 3, 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 5 mmol/L EDTA/Na_3VO_4. After the last wash was removed, PI3K assays were done as described previously (23). Briefly, samples were resuspended in 50 μL of PI3K buffer [20 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.5 mmol/L EGTA], and 10 μg of phosphatidylinositol were added. After 10 min at room temperature, 10 μCi of [^{32}P] ATP and $MgCl_2$ to a final concentration of 20 μmol/L was added. After 10 min at room temperature, lipids were extracted: first extraction with 150 μL of $CHCl_3$/methanol/HCl (10:20:0.2) and 100 μL of pure $CHCl_3$; and second extraction with 80 μL of methanol/1 N HCl (1:1). Samples were spotted on 1% potassium oxalate–treated TLC plates (Analtech, Newark, DE) and developed in $CHCl_3$/methanol/NH_4OH/H_2O (129:114:15:21).

Western Blot Analysis

Cells were lysed in NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L Na_3VO_3, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 25 μg/mL aprotinin, 25 μg/mL trypsin inhibitor, and 25 μg/mL leupeptin] at 4°C. The supernatants were cleared by centrifugation. Protein (30–100 μg) was fractionated on an acrylamide denaturing gel and transferred onto a nitrocellulose membrane (Amersham, Piscataway, NJ) by electroblotting. The membrane was blocked with 5% nonfat dry milk in TBST [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] for 1 h at room temperature or overnight at 4°C and washed in TBST. The membrane was then incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. After washing with TBST for 15 min, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Amersham) for 1 h at room temperature. After further washing in TBST for 15 min, the proteins were detected by the enhanced chemiluminescence system (Amersham).

Apoptosis Assays

Apoptosis assays were done to study the effect of LY294002 (Invitrogen, Carlsbad, CA) on the mutant and wild-type cells. Cells were seeded in 96-well plates. LY294002 was added at indicated concentrations on the next day. Apoptosis assays were done using a DNA fragmentation ELISA kit as described in the manufacturer's protocol (Invitrogen) 72 h later.

Knockdown of p110α Protein Using Small Interfering RNAs

PIK3CA smartpool consisting of four small interfering RNA duplexes (Dharmacon Research, Inc., Lafayette, CO) was used against *PIK3CA*. A scrambled small interfering RNA duplex (Qiagen, Valencia, CA) was used as a control. HCT116, RKO, and CBS cells were plated in six-well plates. The next day, the cells were 60% confluent and transfected with 50 nmol/L small interfering RNA duplexes using LipofectAMINE 2000 (Invitrogen). The cells were lysed for Western blot analyses or subject to DNA synthesis assays or DNA fragmentation assays 72 h later, as described above.

DNA Synthesis Assays

[^{3}H]thymidine incorporation was used to determine cell proliferation of the mutant and wild-type cells after transfection of scrambled or *PIK3CA* small interfering RNA. The cells were seeded in six-well tissue culture plates and grown to 60% confluence. At 72 h after small interfering RNA tarnsfection, the cells were labeled with [^{3}H]thymidine (7 μCi, 46 Ci/mmol; Amersham) for 1 h. DNA was then precipitated with 10% trichloroacetic acid and solubilized in 0.2 mol/L NaOH. The amount of [^{3}H]thymidine that was incorporated was analyzed by liquid scintillation counting in a Beckman LS7500 scintillation counter.

Ecotopic Expression of Wild-Type and Mutant *PIK3CA*

The wild-type and mutant *PIK3CA* (H1047R) cDNAs were kind gifts from Dr. Vogelstein (Johns Hopkins University Medical Institutions), which were then subcloned into a pBabe-based retroviral vector. The 293GP packaging cells (Clontech, Mountain View, CA) were cotransfected with pVSV-G and empty retroviral vector or retroviral vectors containing *PIK3CA* wild-type or mutant cDNA. The viruses were harvested 48 h later and used to infect FET cells. Puromycin (3.0 μg/mL) was used to select infected cells.

Results

Mutations of *PIK3CA* Imparted Constitutive PI3K Activity in Colon Carcinoma Cell Lines

Human colon carcinoma cell lines were screened for *PIK3CA* mutations by direct sequencing of all 20 exons and splice junction regions. Four cell lines were identified as bearing putative gain of function mutations (H1047R in HCT116 and RKO, C378R in TENN, and Q546P in Vaco481) previously described by Samuels et al. as being present in cancer specimens (13). The mutations were verified by sequencing. These mutations were mainly located in the helical or kinase domains. Meanwhile, a cohort of cell lines bearing only the wild-type *PIK3CA* alleles (FET, GEO, CBS, and RCA) was identified and verified by sequencing for comparison to the mutants.

We first examined whether the mutant PI3K was constitutively active. To determine this, the cells were subjected to GFDS with the expectation that if the mutant PI3K was constitutively active, PI3K activity would remain

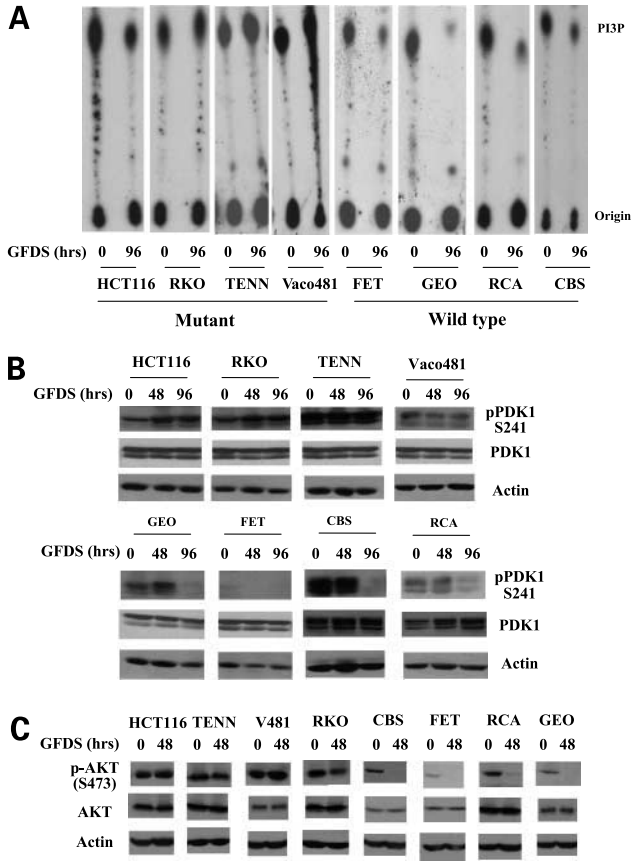

Figure 1. Constitutive activation of PI3K, PDK1, and AKT in mutant *PIK3CA*-bearing colon cancer cells during GFDS. The mutant and wild-type cells were deprived of growth factors or serum for 0, 48, and 96 h after growth to 80% confluence as described in Materials and Methods. **A,** cells were harvested at the indicated times, and the lysates were subjected to PI3K assays as described in Materials and Methods. **B** and **C,** cells were harvested at the indicated times and Western blot analyses were done with pPDK1 (Ser^{241}; *pPDK1 S^{241}*) or pAKT [Ser^{473}; *pAKT (S^{473})*] antibody. Actin was used as a loading control.

high in the mutant cells despite the absence of exogenous growth factors, whereas the removal of growth factors from the wild-type PI3K cells would result in the impairment of its activity. As shown in Fig. 1A, the cell lines carrying the mutant PI3K had relatively higher sustained PI3K activity after 4 days of growth factor and nutrient deprivation, whereas the enzyme activity was significantly reduced in the wild-type cells during GFDS. Taken together with the fact that p110α expression levels did not change under GFDS in both mutant and wild-type cells (data not shown), these results indicated that the mutant cells, but not the wild-type cells, had constitutively active enzyme activity during GFDS. Phosphorylation at Ser^{241} and Ser^{473} was used to reflect the activity of PDK1 and AKT, respectively. The constitutive activation of AKT, which acts downstream of PI3K, further supported the constitutive PI3K activity of the mutant cells observed in Fig. 1A. In contrast, cells with only wild-type PI3K showed rapid decay of phosphorylation of both PDK1 and AKT during GFDS (Fig. 1B and C).

Colon Cancer Cells Harboring Mutant *PIK3CA* Are More Resistant to GFDS-Induced Apoptosis

Because PI3K often plays an important role in cell survival, we next examined whether mutant cells with constitutive PI3K activity would be more resistant to GFDS-induced apoptosis. As shown in Fig. 2, PARP cleavage was significantly increased in the wild-type cells during GFDS, whereas there was almost no change of PARP cleavage in the mutant-bearing cells despite background PARP cleavage at confluence in TENN cells. These results indicated that *PIK3CA* mutations are associated with increased resistance to GFDS-induced apoptosis, suggesting that mutant PI3K enables cells to withstand environmental stresses, such as growth factor and nutrient restriction, that normally would be expected to induce apoptosis in the wild-type cells.

Mutant *PIK3CA*-Bearing Colon Cancer Cells Are Hypersensitive to PI3K Inhibition

Recently, it has been reported that response to Iressa in nonsmall cell lung cancer is closely associated with gain of function mutations of EGFR (24). The data suggest that the sensitivity of the patients to competitive ATP binding site inhibitors is determined by the dependence of the tumors on the gain-of-function mutant EGFR (oncogenic addiction), and that the cells bearing these mutations are, therefore, hypersensitive to EGFR inhibition. Accordingly, we determined whether a similar addiction occurs for colon cancer cells bearing PI3K mutations. Both the wild-type and mutant cells were treated with LY294002, a small molecule that competitively and reversibly inhibits the ATP binding site of PI3K (25). AKT phosphorylation was inhibited by LY294002 in all cell lines, confirming that LY294002 did block PI3K function (Fig. 3A). Induction of apoptosis was then measured after 72 h by a DNA fragmentation ELISA assay. Apoptosis was induced to a much greater degree in the mutant cell lines with the exception of RKO than in the wild-type cell lines except for GEO (for exceptions for RKO and GEO cells, see Discussion; Fig. 3B). These results indicated that, compared with the wild-type cells, the PI3K mutant-bearing cells were by and large hypersensitive to PI3K inhibition.

We then explored the underlying mechanisms of apoptosis induced by LY294002. As shown in Fig. 3C, phosphorylation of FKHR was significantly reduced in HCT116 and TENN cells after LY294002 treatment,

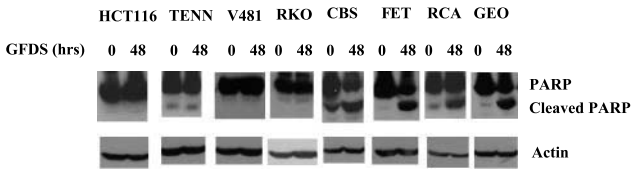

Figure 2. Mutant *PIK3CA* confers resistance to GFDS-induced apoptosis. The mutant and wild-type cells were grown to 80% to 90% confluence, and the cells were then deprived of growth factor or serum for 48 h. Cells were harvested and Western blot analyses were done with PARP antibody. Actin was used as a loading control.

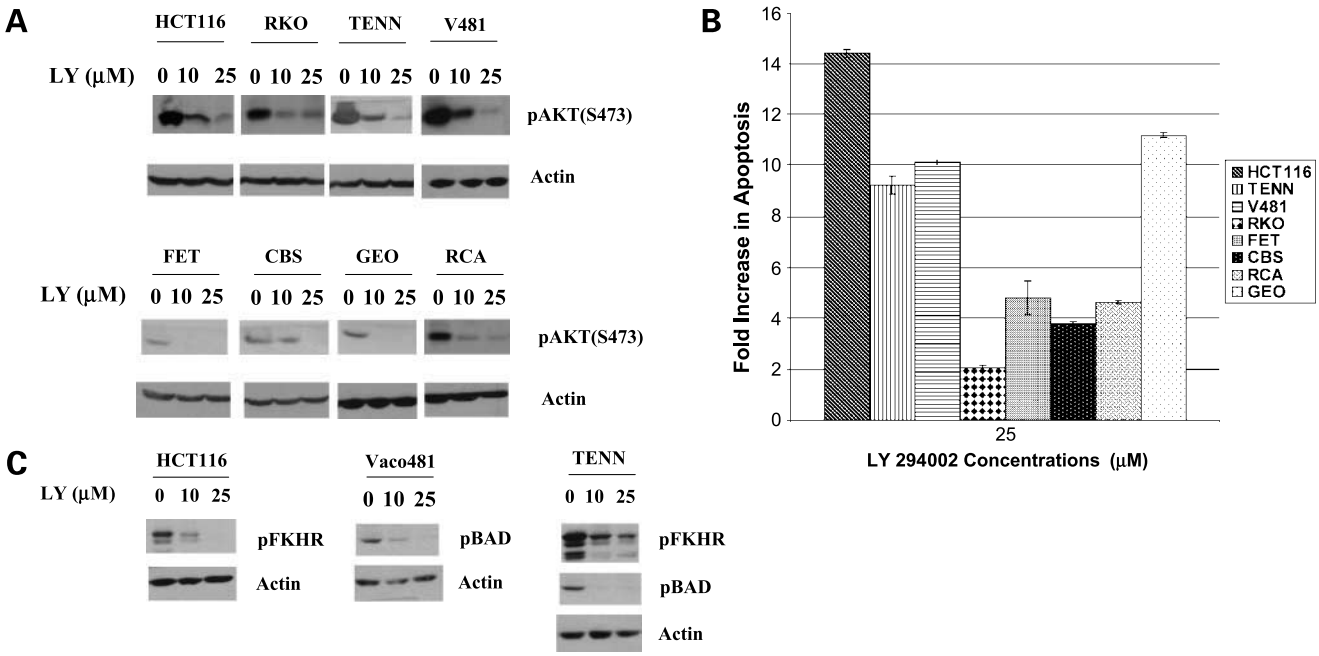

Figure 3. Effect of LY294002 (*LY*) on apoptosis. **A,** cells were grown to 80% confluence and were then deprived of growth factor or serum for 48 h although treated with increasing concentrations of LY294002. Cells were harvested, and Western blot analyses were done with pAKT (Ser^{473}) antibody. Actin was used as a loading control. **B,** cells were seeded in 96-well plates and treated with 25 μmol/L LY294002 for 72 h. DNA fragmentation assays were done as described in Materials and Methods. **C,** cells were grown to 80% confluence and were then deprived of growth factor or serum for 48 h although treated with increasing concentrations of LY294002. Cells were harvested, and Western blot analyses were done with pFKHR or pBAD antibodies. Actin was used as a loading control.

whereas phosphorylation of BAD was greatly inhibited in Vaco481 and TENN cells by LY294002 treatment. These results suggested that reduced phosphorylation of FKHR or BAD, through inhibition of AKT activity, might contribute to LY294002-induced apoptosis in HCT116, TENN, and Vaco481 cells. Interestingly, LY294002 had little effect on either pFKHR or pBAD in RKO cells (data not shown). This may explain why RKO cells were resistant to LY294002 induced apoptosis.

To show that the mutant cells, but not the wild-type cells, are strictly dependent on constitutive activation of PI3K, two of the mutant cell lines, HCT116 and RKO, were challenged with *PIK3CA* small interfering RNAs. Transfection of *PIK3CA* small interfering RNA into both cell lines resulted in significantly reduced expression of p110α with a concomitant decrease of AKT phosphorylation (Fig. 4A), indicating that *PIK3CA* small interfering RNA knocked down p110α expression and inhibited PI3K downstream signaling. As a result, there was increased PARP cleavage and caspase-9 activation in HCT116 p110α knockdown cells compared with the scrambled small interfering RNA transfected control cells. There was, however, no increased PARP cleavage or caspase-9 activation in RKO cells transfected with *PIK3CA* small interfering RNA compared with the scrambled small interfering RNA transfected control cells (Fig. 4A). Consistent with the PARP cleavage Western blot results, DNA fragmentation assays also showed that *PIK3CA* small interfering RNA induced apoptosis in HCT116 cells but not in RKO cells. We further investigated whether knockdown of p110α had any effect on cell proliferation. DNA synthesis was determined 72 h after transfection of *PIK3CA* or scrambled small interfering RNA into the cells. The *PIK3CA* small interfering RNA–transfected cells showed >50% inhibition of DNA synthesis compared with the scrambled small interfering RNA–transfected cells in both HCT116 and RKO cells (Fig. 4C), indicating that both HCT116 and RKO cells are dependent on the PI3K pathway for DNA synthesis and proliferation and that HCT116, but not RKO, cells are dependent on the PI3K pathway for survival. Transfection of *PIK3CA* small interfering RNA into one of the wild-type cell lines, CBS, knocked down p110α expression to the similar level as that in HCT116 or RKO cells (Fig. 4A). However, *PIK3CA* knockdown did not have much effect on apoptosis or DNA synthesis compared with the scrambled small interfering RNA transfection (Fig. 4A–C). Taken together, these results showed that, compared with the wild-type cells, the *PIK3CA* mutant-bearing cells are hypersensitive to PI3K inhibition and display dependence on constitutive PI3K activity for proliferation and/or survival.

Ecotopic Expression of Mutant PI3K Rendered the Wild-Type Cells Resistance to GFDS-Induced Apoptosis

It has been shown that expression of mutant PI3K enhances lipid kinase activity and induces constitutive activation of AKT signaling and oncogenic transformation

in chicken embryo fibroblasts, as well as in NIH3T3 cells (17, 18). However, the response of mutant PI3K-expressing cells to stress conditions has not been addressed. We next determined whether ecotopic expression of one of the ''hotspot'' *PIK3CA* mutants, H1047R, in the wild-type *PIK3CA*-bearing colon cancer FET cells would make the cells more resistant to GFDS-induced apoptosis. Transfection of the wild-type or mutant *PIK3CA* into FET cells resulted in an increase of p110α expression with a concomitant increase of AKT phosphorylation compared with the empty vector-transfected control cells (Fig. 5A). Although the cells transfected with mutant or wild-type *PIK3CA* expressed similar levels of p110α, AKT phosphorylation was slightly higher in the mutant *PIK3CA*–transfected cells than that in the wild-type *PIK3CA*-transfected cells. This was consistent with previous reports showing that mutant p110α conferred higher lipid kinase activity compared with the wild-type protein in NIH3T3 cells and chicken embryo fibroblasts (13, 17, 18). AKT was constitutively phosphorylated during GFDS in the mutant PI3K-transfected cells, whereas AKT phosphorylation was rapidly decreased in the wild-type PI3K or vector-transfected cells (Fig. 5B). Consequently, there was much less PARP cleavage and caspase-3 activation in the mutant PI3K-transfected cells compared with the wild-type PI3K or vector-transfected cells during GFDS (Fig. 5C). These results indicated that mutant PI3K conferred constitutive PI3K activity and resistance to GFDS-induced apoptosis.

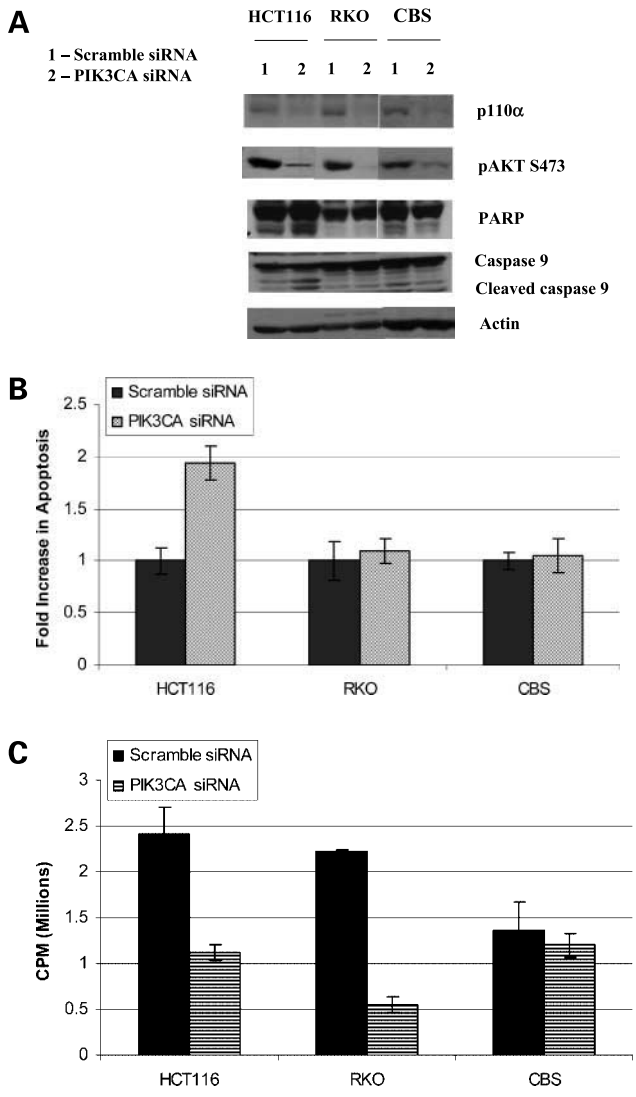

Figure 4. Effect of *PIK3CA* knockdown on DNA synthesis and apoptosis. HCT116, RKO, and CBS cells were seeded in six-well plates and transfected with *PIK3CA* or scrambled small interfering RNA. **A,** cells were harvested 72 h later, and Western blot analyses were done with p110α, pAKT (Ser^{473}), PARP, or caspase-9 antibodies. Actin was used as a loading control. DNA fragmentation assays (**B**) or DNA synthesis assays (**C**) were done on the transfected cells 72 h later.

Discussion

We have identified a subset of colon cancer cell lines with *PIK3CA* mutations. Taken together with the demonstration that *PIK3CA* mutations confer increased PI3K activity and high-transforming activity in NIH3T3 and chicken embryo fibroblasts, respectively, (13, 17, 18) and that Iressa response in nonsmall cell lung cancer is closely associated with gain of function mutations of the EGFR (24), we hypothesized that mutant *PIK3CA* conferred increased growth and survival capacity in colon cancer cells and that mutant *PIK3CA*-bearing cells were highly dependent upon PI3K signaling for their malignant phenotype. The results from this study are consistent with this hypothesis. Mutant *PIK3CA*-bearing cells displayed constitutive activation of PI3K and its downstream mediators during GFDS, which is associated with increased resistance to GFDS-induced apoptosis in the mutant cells relative to the wild-type cells. In addition, the mutant cells were hypersensitive to a potent PI3K inhibitor, LY294002, as reflected by significantly increased apoptosis compared with the wild-type cells and/or hypersensitivity to inhibition of proliferation. *PIK3CA* small interfering RNA dramatically decreased DNA synthesis and induced apoptosis in the mutant cells. These studies indicate that human colon cancer cells with *PIK3CA* mutations display an increased dependence, sometimes called addiction, on the PI3K signaling pathway in cell proliferation and/or survival. This raises the prospect of targeting the PI3K pathway and perhaps targeting the mutant PI3K active site in selective fashion to treat those colon cancer patients with PI3K mutations. Our data provide a biological basis for the treatment of such cancers. Future *in vivo* efficacy studies in relevant animal models will allow for further assessment of the therapeutic potential of small molecular inhibitors against PI3K/AKT signaling pathway in colon cancer.

Similar to the gain-of-function EGFR mutants in lung cancers (24), our studies indicate that the cells expressing mutant *PIK3CA* are hypersensitive to PI3K inhibition and more dependent on aberrant PI3K signaling, therefore suggesting that *PIK3CA* mutations are associated with

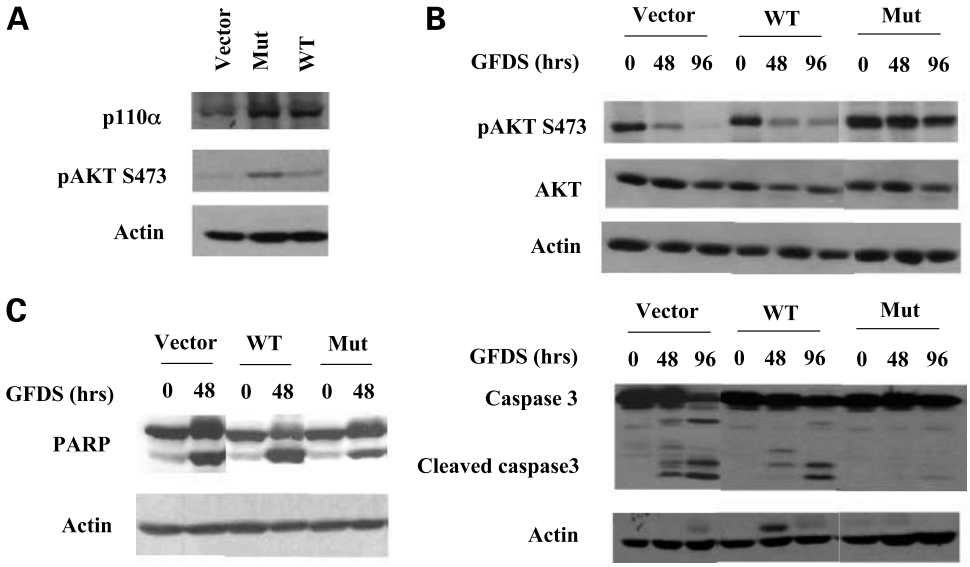

Figure 5. Mutant *PIK3CA* rendered FET cells more resistant to GFDS-induced apoptosis. **A,** FET cells were infected with retroviruses encoding mutant or wild-type *PIK3CA*. After drug selection, the infected cells were harvested and Western blot analyses were done with p110α or pAKT (Ser^{473}) antibodies. Actin was used as a loading control. **B,** vector, wild-type, or mutant *PIK3CA* – infected cells were deprived of growth factor or serum for 48 or 96 h. Cells were harvested and Western blot analyses were done with pAKT (Ser^{473}) or AKT antibodies. Actin was used as a loading control. **C,** vector, wild-type, or mutant *PIK3CA* – infected cells were deprived of growth factor or serum for 48 or 96 h. Cells were harvested, and Western blot analyses were done with PARP or caspase-3 antibodies. Actin was used as a loading control.

oncogenic addiction (26). It has been previously shown that PTEN-deficient tumors had enhanced sensitivity to inhibition of FRAP/mTOR (27) and that PI3K inhibitors selectively inhibited the anchorage-independent growth of ErbB2-overexpressing human mammary carcinoma cells, thereby indicating that those cells displayed an increased dependence on PI3K signaling (28). Thus, there seems to be multiple mechanisms for inactivating the normal regulation of PI3K signaling pathway, including *PIK3CA* mutation. Many investigators have noted from these types of studies that an important role of PI3K in carcinogenesis is implied and suggest that identification of the subset of patients harboring *PIK3CA* abnormalities and developing adequate drugs for targeting the PI3K pathway might be an efficient way to treat this subset of cancers.

There is a recent study demonstrating that there was no difference of PI3K activity and downstream signaling between the wild-type and mutant PIK3CA carrying colon cancer cells under normal cell culture conditions (29). We also observed this in our study. When we examined the PI3K activity or phosphorylation of AKT and PDK1 under normal conditions (GFDS, 0 h), PI3K activity and signaling are very similar between the wild-type and mutant cells. However, there was significant difference in PI3K signaling between the wild-type and mutant cells under GFDS (GFDS, 48 or 96 h; Fig. 1A–C). These results indicated that PI3K activity was sustained in the mutant cells under GFDS, whereas it deceased in the wild-type cells. Sustained PI3K activity under stress conditions would enable mutant cells to survive environmental stresses. Because environmental restriction on growth seems to be common in solid tumors due to inadequate vascularization (20, 30), the ability of PI3K mutant cells to withstand these stresses is considered a key factor in tumor development and progression. Therefore, mutational activation of *PIK3CA* is likely to be one of the mechanisms of tumor progression in colon carcinomas.

Among the mutant *PIK3CA*-expressing cells, only RKO cells were insensitive to apoptosis induced by PI3K inhibition, either through LY294002 treatment (Fig. 3B) or *PIK3CA* small interfering RNA transfection (Fig. 4A and B). However, DNA synthesis and proliferation of RKO cells were associated with hypersensitivity to LY294002 (data not shown) or *PIK3CA* small interfering RNA (Fig. 4C). This reflects the possibility that the PI3K pathway regulates proliferation but not survival in RKO cells. Because LY294002 had no effect on pFKHR or pBAD that plays important roles as target of LY294002-induced apoptosis in other PI3K mutant cells, this result suggested that there were defects in apoptosis pathways in RKO cells. Further studies are needed to determine the mechanism(s) of resistance to LY294002-induced apoptosis in RKO cells. On the other hand, among the wild-type cells, GEO cells were growth factor independent (data not shown) and more sensitive to LY294002-induced apoptosis compared with other wild-type cells (Fig. 3B). GEO cells have been shown to have strong autocrine heregulin activity that results in constitutive ErbB2 activation, which in turn leads to constitutive activation of the PI3K/AKT pathway (31, 32). The PI3K pathway supports both proliferation and cell survival in these cells.

Recently, Samuels et al. did somatic knockouts by eliminating one allele of either wild-type or mutant *PIK3CA* in HCT116 or DLD1 colon cancer cells. They showed that cells bearing only mutant PI3K had reduced dependence on growth factors and were more sensitive to PI3K inhibition by LY294002 (19). We have compared a panel of intact cell lines with the wild-type or mutant *PIK3CA* and native levels of PI3K activity. Because the study by Samuels et al. used somatic *PIK3CA* knockout cells, comparisons of cells bearing two copies of the *PIK3CA* gene were not possible. Thus, the work by Samuels et al. was a comparison of haploid levels of PI3K. Consequently, some of the results observed in the Samuels

study could have reflected haploid insufficiency. In contrast, our study was done with native intact diploid cells, thus reducing the possibility of haploid effects. Our finding that the differential cell survival characteristics between the mutant and wild-type cells under stress conditions, in which the wild-type PI3K is severely down-regulated, provides an explanation for the irrelevance of the wild-type PI3K activity in terms of the advantage provided by the mutant enzyme.

One could argue that there might be other genetic changes contributing to the differences observed between the wild-type and mutant PI3K cells in addition to PI3K status. However, we are asking the question of whether mutant *PIK3CA* imparts properties that can be generalized to the mutant-bearing versus nonmutant-bearing cells, given the many additional genetic changes that can occur in those cells. Our studies indicate that we can distinguish the wild-type and mutant PI3K cells based on their different properties despite the presence of other genetic changes. For example, unlike other mutant cell lines, RKO cells are insensitive to PI3K inhibition–induced apoptosis, probably due to other defects in the apoptosis pathways. However, they are still hypersensitive to PI3K inhibition–induced growth inhibition. Therefore, although there are exceptions in the phenotypes of the wild-type or mutant PI3K cells due to other genetic changes, the overall differences between these two cell types are recognizable. In addition, we show that these properties in the mutant cells can be gained when the nonmutant cells are transfected with mutant *PIK3CA*.

Acknowledgments

We thank David Chervinsky for his expert assistance with the preparation of samples for DNA sequencing.

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Mol Cancer Ther 2007;6:1143-1150.