Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival

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Abstract

The abilities of mutated active RAS proteins to modulate cell survival following exposure to ionizing radiation and small molecule kinase inhibitors were examined. Homologous recombination in HCT116 cells to delete the single allele of K-RAS D13 resulted in a cell line that exhibited an ~75% reduction in basal extracellular signal-regulated kinase 1/2, AKT, and c-*jun*-NH_2-kinase 1/2 activity. Transfection of cells lacking K-RAS D13 with H-RAS V12 restored extracellular signal-regulated kinase 1/2 and AKT activity to basal levels but did not restore c-*jun*-NH_2-kinase 1/2 phosphorylation. In cells expressing H-RAS V12, radiation caused prolonged intense activation of AKT. Inhibition of H-RAS V12 function, blockade of phosphatidylinositol 3-kinase (PI3K) function using small interfering RNA/small-molecule inhibitors, or expression of dominant-negative AKT abolished radiation-induced AKT activation, and radiosensitized these cells. Inhibition of PI3K function did not significantly radiosensitize parental HCT116 cells. Inhibitors of the AKT PH domain including perifosine, SH-(5, 23-25) and ml-(14-16) reduced the plating efficiency of H-RAS V12 cells in a dose-dependent fashion. Inhibition of AKT function using perifosine enhanced radiosensitivity in H-RAS V12 cells, whereas the *SH* and *ml* series of AKT PH domain inhibitors failed to promote radiation toxicity. In HCT116 H-RAS V12 cells, PI3K, PDK-1, and AKT were membrane associated, whereas in parental cells expressing K-RAS D13, only PDK-1 was membrane bound. In H-RAS V12 cells, membrane associated PDK-1 was phosphorylated at Y373/376, which was abolished by the *Src* family kinase inhibitor PP2. Inhibition of PDK-1 function using the PH domain inhibitor OSU-03012 or using PP2 reduced the plating efficiency of H-RAS V12 cells and profoundly increased radiosensitivity. OSU-03012 and PP2 did not radiosensitize and had modest inhibitory effects on plating efficiency in parental cells. A small interfering RNA generated against PDK1 also radiosensitized HCT116 cells expressing H-RAS V12. Collectively, our data argue that molecular inhibition of AKT and PDK-1 signaling enhances the radiosensitivity of HCT116 cells expressing H-RAS V12 but not K-RAS D13. Small-molecule inhibitory agents that blocked stimulated and/or basal PDK-1 and AKT function profoundly reduced HCT116 cell survival but had variable effects at enhancing tumor cell radiosensitivity. [Mol Cancer Ther 2005;4(2):257–70]

Introduction

Ionizing radiation is used as a primary treatment for many types of carcinoma. Although it has been appreciated for many years that radiation causes cell death, it has only recently become accepted that radiation has the potential to enhance proliferation in the surviving fraction of cells (1, 2). We and others have observed that exposure of carcinoma cells to low radiation doses causes an initial early activation of growth factor receptors in the plasma membrane, followed by secondary receptor activation that is dependent upon paracrine/autocrine growth factors (3, 4). Receptor activation enhances the activities of RAS family molecules that signal to cause activation of multiple intracellular signal transduction pathways. Secondary activation of intracellular signal transduction pathways by growth factors and radiation has been correlated to altered expression of cell cycle regulatory proteins and may, under certain circumstances, promote cell proliferation (5, 6).

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Growth factors interact with plasma membrane receptors, which transduce signals through the membrane to its inner leaflet (7–14). Growth factor signals, via guanine nucleotide exchange factors, can increase the amount of GTP bound to membrane-associated GTP binding proteins, including RAS (15, 11). There are three widely recognized isoforms of RAS: Harvey, Kirsten, and Neuroblastoma (16). GTP-RAS can interact with multiple downstream effector molecules including the Raf-1 protein kinase and the phosphatidyl inositol 3-kinase (PI3K) lipid kinase. Receptor-stimulated guanine nucleotide exchange of ''RAS'' to the GTP-bound form permits Raf-1 and P110 PI3K to associate with RAS, resulting in kinase translocation to the plasma membrane environment where activation of these kinases, via complex mechanisms, takes place. RAS contains a GTPase activity that converts bound GTP to GDP resulting in inactivation of the RAS molecule. PI3K enzymes are also translocated to the plasma membrane environment via the P85 SH2 domain interaction with phosphorylated tyrosine residues on adaptor proteins and growth factor receptors (e.g., GAB2, IRS-1, and ERBB3; ref. 17).

Mutation of RAS results in a loss of GTPase activity, generating a constitutively active RAS molecule that can lead to elevated activity within downstream signaling pathways. Approximately one third of human cancers have RAS mutations, primarily the K-RAS isoform, that also lead to a radio-protected phenotype (15, 18). Of note is that some studies suggest that K-RAS and H-RAS have different but overlapping signaling specificities to downstream pathways as determined by *in vitro* cell-based studies and in animal knock-out models. Specifically, mutant K-RAS is thought to preferentially activate the Raf-1/extracellular regulated kinase (ERK1/2) pathway, whereas mutant H-RAS is believed to preferentially activate the PI3K/AKT pathway (19–21). It has been argued that ERK1/2 and PI3K signaling downstream of K-RAS and H-RAS, respectively, can in turn control cell growth and cell survival following exposure to multiple growth factors (e.g., epidermal growth factor; refs. 3, 9, 10).

Loss of mutant K-RAS expression has been shown in HCT116 cells to abolish tumor formation in athymic mice and enhance radiosensitivity (22, 23). The findings presented in these studies were linked to reduced expression of the paracrine growth factor epiregulin. Repeated irradiation of tumor cells can also increase expression of transforming growth factor α and ERBB1, and radiation is known to promote the cleavage and release of transforming growth factor α from tumor cells in an ERK1/2-dependent fashion (24–26). Increased proliferative rates and poor prognosis of carcinomas *in vivo* have also been correlated with increased expression of ERBB1 (27).

Recent studies in rodent fibroblasts argue that H-RAS and K-RAS differentially alter radiosensitivity, with mutated active K-RAS promoting radiation-sensitization, through a process linked to activation of the p38 mitogen-activated protein kinase pathway (28). However, the roles of different RAS isoforms and the signaling pathways used by radiation to alter radiosensitivity of isogenic human carcinoma cells are unknown. The present studies were undertaken to determine whether constitutively active mutant K-RAS or mutant H-RAS differentially alter the signaling properties and radiosensitivity of HCT116 colon carcinoma cells.

Materials and Methods

Materials

Anti-Phospho-ERK1/2, anti-total-ERK2, anti-β-actin antibodies, and protein A/G-conjugated agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Phospho-JNK1/2, anti-Phospho-Ser473-AKT, anti-Phospho-Thr308-AKT, anti-total-AKT, anti-Phospho-Ser9-GSK3 β, anti-GSK3 β, anti-Phospho-Ser241- PDK1, anti-Phospho-Tyr373/376-PDK1, anti-total-PDK1, and Anti-Phospho-p38 antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies to detect H-RAS and heregulin were from Oncogene Research Products (Cambridge, MA). Antibodies to detect ERBB3 and for immunoprecipitation of ERBB3 were from Neomarkers Lab Vision Co. (Fremont, CA). Antibodies anti-PI3K p85 and anti-PI3K p110 α were from Upstate Biotechnology (Lake Placid, NY). The selective inhibitor of farnesyltransferase FTI277 and the PI3K inhibitor SH-5 were from Calbiochem (San Diego, CA). Perifosine was kindly provided by Dr. Janet Dancey, Cancer Treatment and Evaluation Program, National Cancer Institute, Bethesda, MD.

Generation of HCT116 Cell Lines without Mutant K-RAS and Expressing Mutant H-RAS

HCT116 mutant K-RAS-deleted cells were generated by homologous deletion of the mutant *K-RAS* allele as described (22, 29). A plasmid expressing mutant active H-RAS (H-RAS V12) was kindly provided by the laboratory of Dr. M. Wigler (Cold Spring Harbor, NY). A plasmid expressing dominant-negative AKT (AKT1-S473A/T308A mutant) was kindly provided by Dr. R.A. Roth (Stanford University, Palo Alto, CA). HCT116 cell lines were transfected by electroporation at 600 V for 60 milliseconds using a Multiporator Eppendorff (Hamburg, Germany) with control plasmids (''C2'' cells) or plasmids to express H-RAS V12 (''C10'' and ''C3'' cells) or dominant-negative AKT. Pools of transfected cells were obtained by puromycin (RAS) or neomycin (AKT) selection and individual colonies isolated and then characterized.

Culture of HCT116 Cell Lines

Asynchronous carcinoma cells were cultured in DMEM media supplemented with 10% (v/v) FCS at 37°C in 95% (v/v) air/5% (v/v) CO_2. Cells were plated at a density 3×10^3 cells/cm^2 plate area and all cells were plated from log phase cultures. For radiation-induced activations of protein kinases, cells were cultured for 4 days in this media, and for 24 hours before irradiation were cultured in serum-free DMEM medium. For colony formation assays, cells were plated at low density (250–2,000 cells per dish) and 24 hours after plating, for the 24 hours before irradiation, were

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cultured in serum-free DMEM medium. Cells were irradiated (1–4 Gy) or as indicated in the text: media was replaced with serum containing media 24 hours after radiation exposure. Ten to 14 days after exposure, plates were washed in PBS, fixed with methanol and stained with a filtered solution of crystal violet (5% w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount TM plate reader. Data presented is the arithmetic mean (±SE) from both counting methods from multiple studies.

Exposure of Cells to Ionizing Radiation and Cell Homogenization

Cells were cultured as described above. As indicated, 1 hour before irradiation cells were treated with either vehicle (DMSO), the PKB inhibitor perifosine (1, 3, or 10 μmol/L), the novel PDK1 inhibitor OSU-03012 (1, 3, or 10 μmol/L), the Src family kinase-inhibitor PP2 (3, 10, or 30 μmol/L; D-3-deoxy-2-*O*-methyl-myo-inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate] SH-5). Treatment was from a 100 mmol/L stock solution and the maximal concentration of vehicle (DMSO) in media was 0.01% (v/v). Cells were irradiated using a ^{60}Co source at dose rate of 1.8 Gy/min. Cells were maintained at 37°C throughout the experiment except during the irradiation itself. Zero time is designated as the time point at which exposure to radiation ceased. After radiation treatment cells were incubated for specified times followed by aspiration of media and immediately homogenized in 1 mL SDS-PAGE lysis buffer [5% (w/v) SDS, 40% (v/v) glycerol, 250 mmol/L Tris-HCl, 10% (v/v) 2-mercaptoethanol]. Homogenates were sonicated, boiled for 10 minutes, the protein concentration determined by Bradford assay (Coomassie Protein Assay Kit, Pierce Biotechnology, Rockford, IL), and stored frozen (−20°C) before use.

SDS-PAGE and Western Blotting

Depending on the protein to be studied, a volume of homogenate containing 10, 20, or 40 μg of total protein was loaded in 12% (w/v) acrylamide gels and subjected to SDS-PAGE. Gels were transferred to nitrocellulose and Western blotted using specific antibodies. Blots were developed using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) and Kodak X-ray film, or using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) which has 3-log detection sensitivity over conventional enhanced chemiluminescence immunoblotting. This machine uses dye-conjugated secondary antibodies that fluoresce in IR light as a detection system rather than an enzymatic chemiluminescent reaction. Secondary antibodies are available that fluoresce red or green, which are detected on two separate channels in/by the machine (Molecular Probes, Eugene, OR and Rockland Immunochemicals, Gilbertsville, PA). Thus, immunoblots to detect multiple proteins of different masses can be digitally scanned/probed on the same piece of nitrocellulose at the same time. Densitometric analysis for enhanced chemiluminescence immunoblots and reverse transcription-PCR analyses was done using a Fluorochem 8800 Image System and the respective software (Alpha Innotech Co., San Leandro, CA) and band densities were normalized to that of β-actin in the same sample and expressed as a percentage of the respective control in each experiment as indicated in the legends to figures. Blots were digitally scanned using Adobe Photoshop 7, their color removed, and figures created in Microsoft PowerPoint.

Cell Death Assays: Wright Giemsa for Apoptosis

Cells were plated at 5×10^4 cells per well in 12-well plates, and 24 hours later they were serum-starved. Twenty-four hours later, the plates were mock exposed or irradiated at 1 or 4 Gy and harvested 96 hours after irradiation by trypsinization followed by centrifugation onto glass slides (cytospin) at 800 rpm for 10 minutes. The cells were fixed and stained with a commercial kit (Diff-Quik) following the instruction of the manufacturer (Dade Behring AG, Düdingen, Switzerland). Randomly selected fields of stained cells (~200 cells per field, n = 5 per slide) were counted for apoptotic nuclear morphology.

Membrane Preparation from HCT116 Cells

Cells were cultured as described above. Twenty-four hours after serum withdrawal/starvation, cells were scraped into 10 mL of 1 mmol/L $NaHCO_3$ (pH 7.4), 1 mmol/L Na pyrophosphate, 1 mmol/L Na orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride and incubated for 1 hour at 4°C. Crude membrane preparations were prepared as described in refs. (30) and (31), by sucrose density overlayer centrifugation.

Transfection of HCT116 Cells with Small Interfering RNA Molecules

RNA interference or gene silencing for down-regulating the expression of PDK1 and PI3K was done using validated target sequences designed by Ambion, Inc. (Austin, TX). For transfection, 100 nmol/L of the annealed siRNA targeting PDK1 (sense GGCUCUUUUUCCACGGUUGtt and antisense CAACCGUGGAAAAAGAGCCtt), PI3K α (sense GGAUAGACAAGUGACUGACtt and antisense GUCAGUCACUUGUCUAUCCtt), PI3K β (sense GGAGGAGAACAGAGCCAAGtt and antisense CUUGGCUCUGUUCUCCUCCtt), the positive control doubled-stranded small interfering RNA (siRNA) targeting GAPDH or the negative control (a ''scrambled'' sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used. The small RNA sequences for PDK1, the positive control or the negative control were transfected in wild-type (WT) or C10 cells by electroporation at 600 V during 60 microseconds and the cells were cultured in regular medium. For interference of PI3K, the small RNA sequences for PI3Kα and PI3Kβ were cotransfected at 100 nmol/L each, and the positive or negative controls were transfected in C10 cells by electroporation at 600 V during 60 microseconds and the cells were cultured in regular medium. Forty-eight hours after transfection, the cells were plated for colony formation assays as described above.

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Data Analysis

Comparison of the effects of treatments was done using one-way ANOVA and a two-tailed t test. Differences with a $P < 0.05$ were considered statistically significant. Experiments shown, except where indicated, are the means of multiple individual points from multiple separate experiments (±SE).

Results

Generation and Characterization of WT/Parental, Mutant K-RAS-Deleted, and H-RAS V12 Expressing HCT116 Cells

We obtained HCT116 cell lines that had been genetically manipulated with the allele expressing K-RAS D13 deleted by homologous recombination, whereas leaving the other allele of WT K-RAS intact (termed hereafter ''mutant K-RAS-deleted cells''; refs. 22, 29). HCT116 cell lines were stably transfected to express H-RAS V12 (termed hereafter ''C10'' or ''C3'' cells). Colonies were initially characterized and selected for further study based on total RAS mRNA and protein expression, which was within ~2- to 3-fold of those levels found in parental cells (Fig. 1A).

Differential Regulation of Radiation-Induced Signaling Pathway Activation by Mutant K-RAS and Mutant H-RAS

Loss of K-RAS D13 expression significantly reduced basal ERK1/2 and AKT activity and abolished c-jun-NH_2-kinase (JNK) 1/2 activity in HCT116 cells (Fig. 1B). Transfection of K-RAS D13-deleted cells with H-RAS V12 restored basal ERK1/2 and AKT activity to approximately those levels found in WT cells. However, basal JNK1/2 activity was not restored by expression of H-RAS V12 (Fig. 1B). In contrast to recent studies in rodent fibroblasts transfected to express mutated active H-RAS and K-RAS proteins (28), basal p38 activity was not detected in any of the HCT116 carcinoma cell lines examined (Fig. 1B).

H-RAS V12 Expression Promotes Radiation-Induced PI3K/AKT Pathway Activation H-RAS V12 (C10) Cells

H-RAS V12 has been proposed to enhance PI3K signaling, and we examined the modulation of AKT activity following a 1-Gy radiation exposure in the H-RAS V12 (C10) transfected HCT116 cell line. In parental HCT116 cells, AKT was modestly activated shortly following radiation exposure (5–30 minutes), several hours after exposure (1–4 hours) but was not activated at later times (6–24 hours; Fig. 1C). Activation of AKT by radiation was abolished in mutant K-RAS D13-deleted cells. In mutant K-RAS D13-deleted cells expressing H-RAS V12, AKT was strongly activated by radiation, above the values found in parental cells, which seemed to occur in three phases, 5 to 30 minutes, 1 to 4 hours, and 6 to 24 hours after exposure.

Ionizing radiation promotes cell death by multiple mechanisms; in general, colony formation assays are believed to be the best reflection of cell survival after radiation exposure. Thus, the modulation of radiosensitivity by signaling pathway inhibitors after a 1-Gy exposure was determined in parental HCT116 cells. In general agreement with our findings and those published by Ries et al. (23), incubation of parental HCT116 cells with the mitogen-activated protein kinase kinase 1/2 inhibitors U0126 and PD184352 significantly reduced survival in colony formation assays (Table 1, data not shown). In contrast, an inhibitor of PI3K had no significant effect on the survival of parental HCT116 cells. Additional studies then examined cell survival in mutant K-RAS D13-deleted cells expressing H-RAS V12 (H-RAS V12 cells, C10, and C3). In H-RAS V12 (C10 and C3) cells, inhibition of PI3K using either LY294002 or siRNA molecules targeted against PI3 K p110α and p110β blocked AKT activation and caused a large reduction in colony formation after irradiation whereas inhibition of mitogen-activated protein kinase kinase 1/2 had no effect on cell survival (Table 1; Fig. 1C).

AKT Activation Promotes Survival in Irradiated H-RAS V12 Cells

Cells lacking expression of an activated RAS protein (C2) were found to be more radiosensitive than either parental (WT) cells or H-RAS V12 (C10) cells, with H-RAS V12 cells being more radio-resistant than parental cells expressing K-RAS D13 (Table 1; Fig. 2A). Several pathways exist downstream of PI3K that could mediate radiation resistance in H-RAS V12 cells, including the PDK-1/AKT pathway that has been linked to PI3K-dependent protection from cytotoxic drug lethality. Some investigators have argued that PI3K signaling may protect cells from ionizing radiation via AKT-independent pathways (30–34). Thus, we next investigated whether inhibition of PDK-1 and AKT function altered HCT116 cell survival after radiation exposure.

Treatment of HCT116 H-RAS V12 (C10) cells with a commercially available plextrin homology domain inhibitor of AKT, SH-5, at concentrations above the reported IC_{50} values for AKT inhibition (IC_{50}, ~5 μmol/L), reduced the plating efficiency of HCT116 cell lines but did not significantly modulate radiosensitivity after a 1- to 4-Gy exposure (Fig. 2B, C, and D; data not shown). The novel therapeutic drug perifosine has also been reported to be a potent inhibitor of AKT signaling by blocking AKT PH domain function (35). Perifosine reduced the plating efficiency and radiosensitized H-RAS V12 cells (Fig. 2C and D). In general agreement with data using perifosine, expression of dominant-negative AKT radiosensitized H-RAS V12 (C10) cells (Fig. 2D). LY294002, SH-5, perifosine, and dominant-negative AKT all variably blunted basal AKT activity and blunted radiation-induced activation of AKT 6 hours after exposure (Fig. 2E). However, perifosine but not SH-5 also noticeably reduced basal AKT activity (for up to 24 hours). This data suggests that a portion of the radioprotective effect of H-RAS V12 expression in HCT116 cells was mediated by enhanced AKT signaling. These findings also argue that the inability of SH-5 to radiosensitize H-RAS V12 cells may be due to its relative lack of inhibition of basal AKT activity. Further studies then

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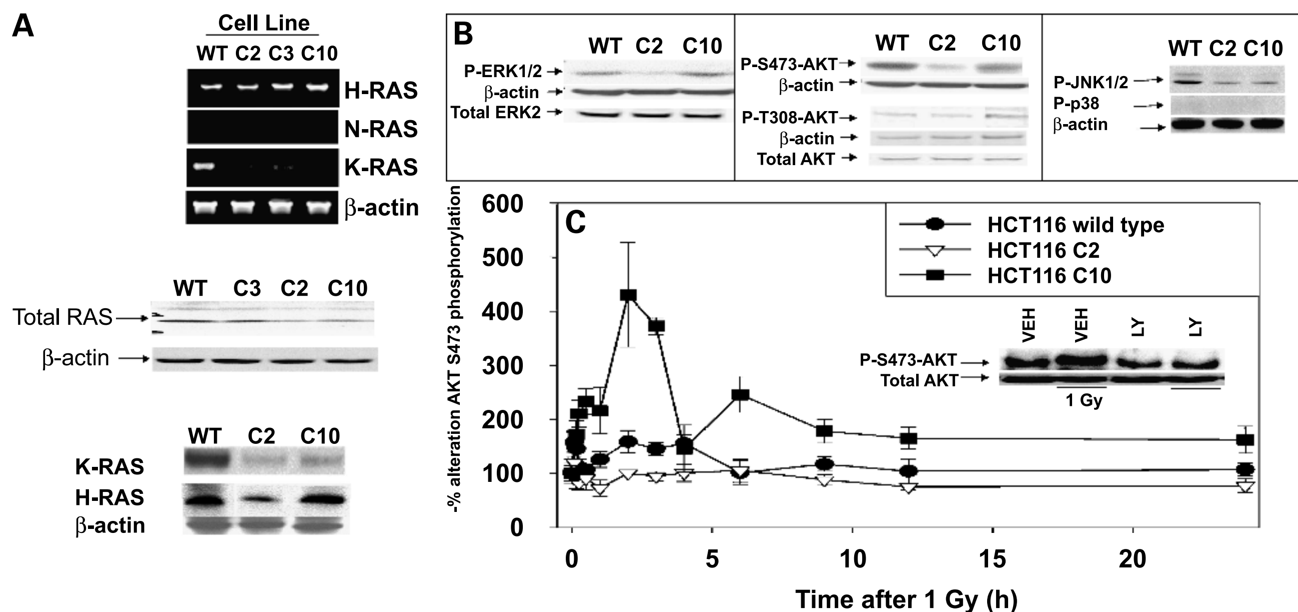

Figure 1. Basal levels of P-ERK1/2, P-AKT S473, P-AKT T308, and P-JNK1/2, and the phosphorylation of P-AKT S473 after a 1-Gy radiation exposure in HCT116 cell lines. **A,** levels of mRNA and protein for H-RAS, K-RAS, and N-RAS in HCT116 cell clones. *Top,* mRNA levels of H-RAS, K-RAS, N-RAS, and β-actin were determined by reverse transcription-PCR using the specific primers as described in Materials and Methods. Representative experiment (n = 4). *Middle,* expression of total RAS protein in WT, C2 (mutant K-RAS deleted), C3 (mutant K-RAS deleted expressing H-RAS V12), and C10 (mutant K-RAS deleted expressing H-RAS V12). *Bottom,* expression of K-RAS and H-RAS proteins determined by immunoblotting using RAS isoform–specific antibodies from a representative experiment showing data from WT, C2 (mutant K-RAS deleted), and C10 (mutant K-RAS deleted expressing H-RAS V12). **B,** phosphorylation (activity) of protein kinases was determined by immunoblotting using specific antibodies for the phosphorylated forms of ERK1/2, AKT S473/T308, JNK1/2, and p38 in parental HCT116 cells (WT), mutant K-RAS-deleted cells (C2), or mutant K-RAS-deleted cells expressing mutant H-RAS V12 (C10). Total β-actin, ERK2, and AKT1/2 protein expression was blotted in the same or parallel sample membrane as a loading control. **C,** alteration of AKT S473 phosphorylation 0 to 24 h after a 1-Gy radiation exposure in WT, C2, and C10 cells. Densitometry values of P-AKT S473 were normalized with respect to total β-actin protein expression and expressed as a percentages of AKT phosphorylation in WT cells at t = 0. *Inset,* LY294002 (1 μmol/L) inhibits AKT P-S473 phosphorylation 6 h after radiation exposure. Representative experiment (n = 2). *Points,* mean from four experiments; *bars,* ±SE.

examined a range of chemically distinct AKT PH domain inhibitors (36). H-RAS V12 cells were treated with increasing doses of SH-23, SH-24, SH-25, ml-14, ml-51, and ml-16 and irradiated (1–4 Gy). SH-(23-25) and ml-(14-16) reduced AKT activity in a similar manner to SH-5 (Fig. 2E, data not shown). Perifosine seemed to cause a more intense and prolonged inhibition of P-AKT levels than SH-5 (Fig. 2E), which may explain our findings in Fig. 2B showing that perifosine but not SH-5 is a radiosensitizer. SH-(23-25) and ml-(14-16) reduced the plating efficiency of H-RAS V12 cells in a dose-dependent fashion (Fig. 3A). However, in a similar fashion to our data using SH-5, SH-(23-25), and ml-(14-16) did not radiosensitize H-RAS V12 cells and parental HCT116 cells (Fig. 3B, data not shown).

Table 1. Inhibition of MEK1/2 radiosensitizes parental (wild type) HCT116 cells and inhibition of PI3K radiosensitizes H-RAS V12 (C10) cells

Treatment	VEH + 1 Gy	U0126 + 1Gy	LY294002 + 1 Gy	siSCR + 1 Gy	siPI3Kα/β + 1 Gy
Parental (wild type)	0.69 ± 0.08	0.43 ± 0.07*	0.69 ± 0.10	0.67 ± 0.08	0.71 ± 0.09
K-RAS D13$^-$ (C2)	0.48 ± 0.07	0.43 ± 0.06	0.39 ± 0.06*	NP	NP
H-RAS V12 (C10)	0.89 ± 0.08	0.77 ± 0.10	0.30 ± 0.07*	0.88 ± 0.08	0.57 ± 0.07*
H-RAS V12 (C3)	0.91 ± 0.07	NP	0.42 ± 0.06*	NP	NP

NOTE: For assays using the inhibitors LY294002 and U0126: Parental (wild type) HCT116 cells and H-RAS V12 (C10 and C3) cells were plated in parallel to those described in Fig. 5 legend and serum starved for 24 hours. Thirty minutes before irradiation, cells were treated with vehicle (DMSO), PI3K inhibitor (LY294002, 1 μmol/L), or the MEK1/2 inhibitor (U0126, 1 μmol/L). Cells were subjected to a 1-Gy or to a mock exposure, as described in Materials and Methods. For assays using small interfering RNA (siRNA): H-RAS V12 (C10) cells were plated and a siRNA molecule against PI3K p110α/β (siPI3K), a scrambled PI3K siRNA (siSCR) as described in Materials and Methods. Forty-eight hours after transfection, cells were replated for colony formation assays and 24 hours after plating for these assays, serum starved for 24 hours, and then irradiated (1 Gy). Media containing serum was added 24 hours after exposure as indicated in Materials and Methods. For both assays, colonies were counted 10 to 14 days later as described in Materials and Methods. The numbers of colonies were expressed as % respective mock exposed group (defined as 1.00). Data are the means of six separate dishes from three separate experiments ± SE.
Abbreviation: NP, not performed.
*P < 0.05 less than corresponding vehicle/siSCR–treated cell value.

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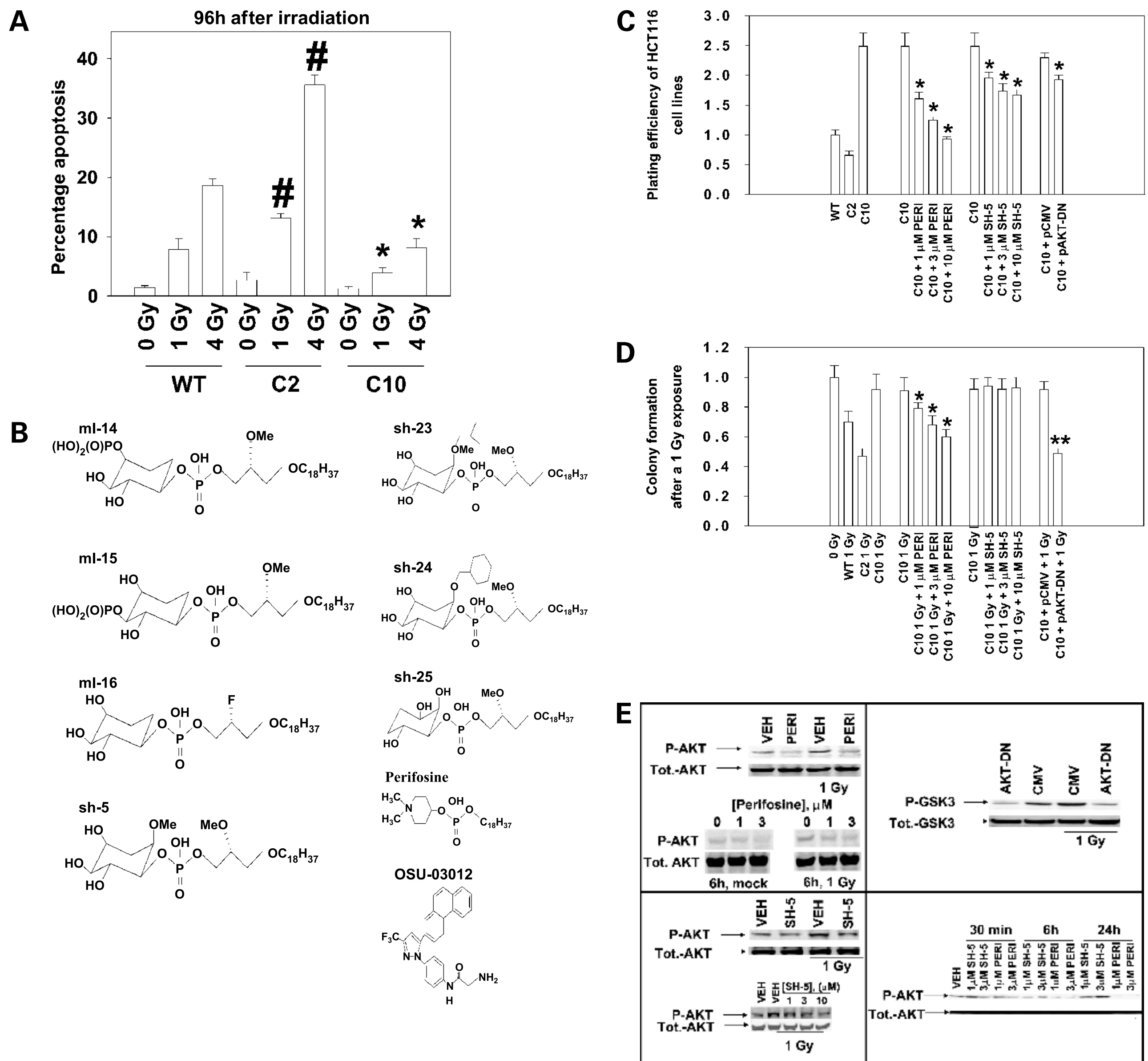

Figure 2. Expression of H-RAS V12 causes a greater radio-protective/survival effect than K-RAS D13 in HCT116 cells. **A,** percentage of apoptotic cells after 0, 1, or 4 Gy in parental HCT116 cells (WT), mutant K-RAS-deleted cells (C2), or in H-RAS V12 cells (C10). Cells were serum-starved and 24 h later were mock exposed or irradiated (1 or 4 Gy). After 96 h, cells were harvested, attached to a slide, fixed, and stained with Wright-Giemsa stain and apoptotic cells were counted under microscope as described in Materials and Methods. *Columns*, % apoptotic cells ($n = 4$); *bars,* ±SE. #, $P < 0.05$ greater than parallel value in parental/WT cells; *, $P < 0.05$ less than parallel value in parental/WT cells. **B,** chemical structures of the AKT inhibitor perifosine, the SH series, and ml series of AKT inhibitors and the PDK-1 inhibitor OSU-03012. **C,** plating efficiency of parental (WT), mutant K-RAS D13-deleted cells (C2), or H-RAS V12 cells (C10) was determined as described in Materials and Methods. For drug treatments, HCT116 cells expressing H-RAS V12 (C10 cells) were serum starved for 24 h before radiation exposure (1 Gy) and treated 30 min before exposure with vehicle, perifosine (*PERI*; 1, 3, or 10 μmol/L) or SH-5 (1, 3, or 10 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. **D,** numbers of colonies were expressed as a fraction of the respective mock irradiated cells. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value; **, $P < 0.05$ less than value treated with 1 μmol/L perifosine. In parallel assays, HCT116 cells expressing H-RAS V12 (C10 cells) were transfected with vector control plasmid or plasmid to express dominant-negative AKT, as described in Materials and Methods. Pools of transfected cells were plated for colony formation assays and irradiated (1 Gy) as described in Materials and Methods. *Columns,* means of three separate dishes from three separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding C10 cell or C10 control plasmid cell value. **E,** *top left,* cells were treated with perifosine (*PERI*, 1 μmol/L; or as indicated) 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and processed to determine P-AKT S473 phosphorylation. *Top right,* pools of cells transfected with either control vector or vector to express dominant negative AKT were irradiated, isolated 6 h after irradiation, and processed to determine GSK3 (S9/S20) phosphorylation. *Bottom left,* cells were treated as indicated with SH-5 (1 or 0 – 10 μmol/L) 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and processed to determine P-AKT S473 phosphorylation. *Bottom right,* cells were treated with vehicle (*VEH*), perifosine (*PERI*, 1 – 3 μmol/L) or SH-5 (1 – 3 μmol/L) for 30 min, and 6 and 24 h, at which time all cells were isolated and processed to determine P-AKT S473 phosphorylation (vehicle control was for cells treated with DMSO for 24 h). Representative experiment ($n = 3$).

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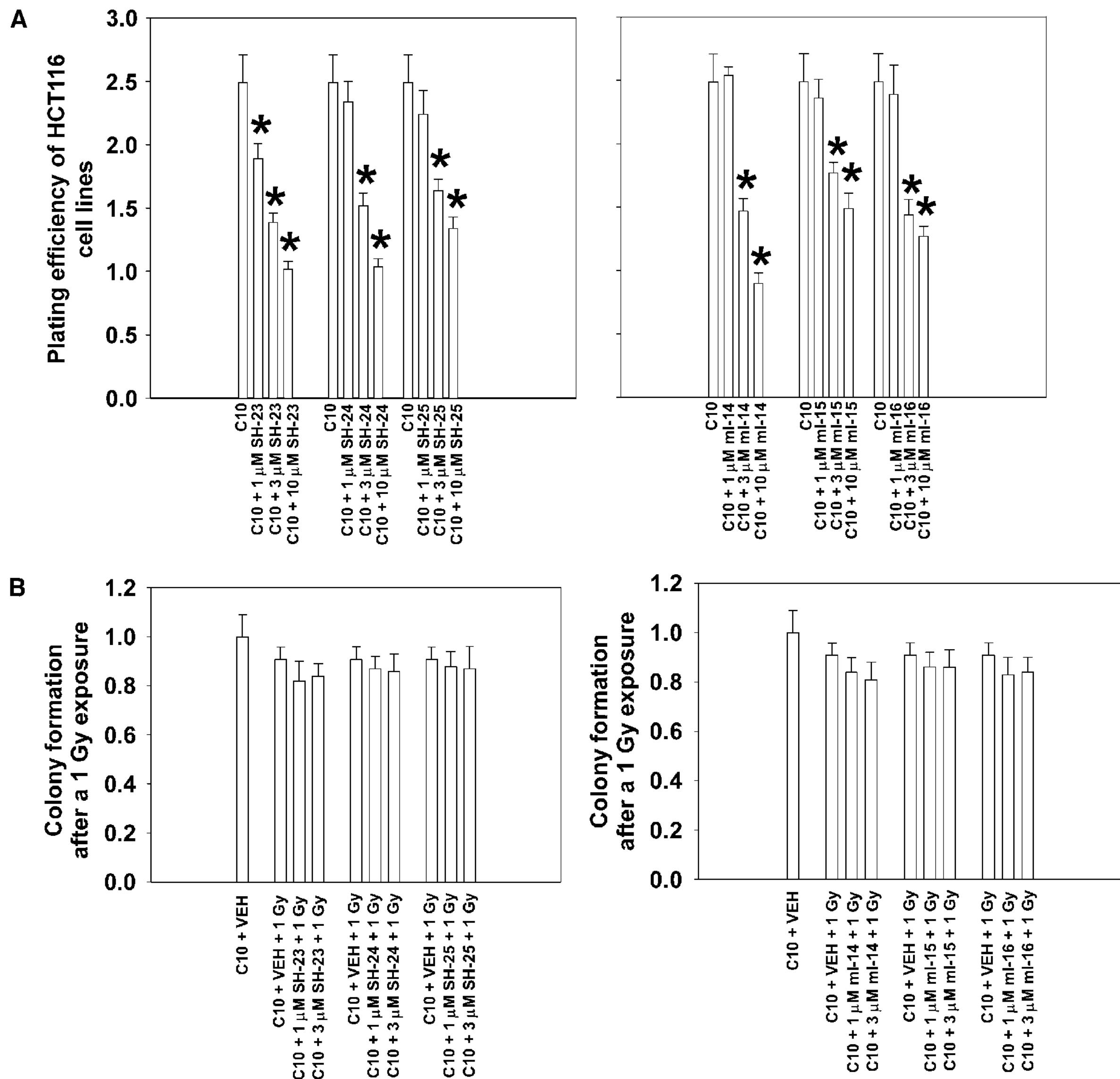

Figure 3. The AKT PH domain inhibitors SH-(23-25) and ml-(14-16) reduce the plating efficiency of H-RAS V12 (C10) cells but do not alter cellular radiosensitivity. **A,** HCT116 cells expressing H-RAS V12 (C10 cells) were serum starved for 24 h before mock radiation exposure and treated 30 min before mock exposure with vehicle, SH-(23-25) (1, 3, or 10 μmol/L) or ml-(14-16) (1, 3, or 10 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. Plating efficiency of H-RAS V12 (C10) cells exposed to the various drugs with mock radiation exposure. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value. **B,** HCT116 cells expressing H-RAS V12 (C10 cells) were serum starved for 24 h before radiation exposure (1 Gy) and treated 30 min before exposure with vehicle, SH-(23-25) (1, 3, or 10 μmol/L) or ml-(14-16) (1, 3, or 10 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. Radiosensitivity of H-RAS V12 (C10) cells exposed to the various drugs with a 1-Gy radiation exposure. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value.

H-RAS V12 (C10) Cells Constitutively Localize PI3K (p110 and p85) and AKT in Their Plasma Membranes: PI3K Localization Is H-RAS V12 Dependent

A possible explanation for our findings in Fig. 1 is that radiation-induced activation of AKT in H-RAS V12 cells is dependent upon constitutive translocation of PI3K and the downstream effectors PDK-1 and AKT into the plasma membrane environment. In agreement with this possibility, high levels of PI3K (p85 and p110 subunits) were detected in unstimulated plasma membranes from H-RAS V12 cells, with much lower amounts of these proteins detected in membranes from both WT and mutant K-RAS-deleted cells (Fig. 4A). To determine whether translocation of PI3K into the plasma membrane environment was H-RAS V12-dependent, H-RAS V12 (C10) cells were treated with a farnesyltransferase inhibitor (FTI). H-RAS translocation into membranes is dependent upon lipid modification of the H-RAS protein, including the

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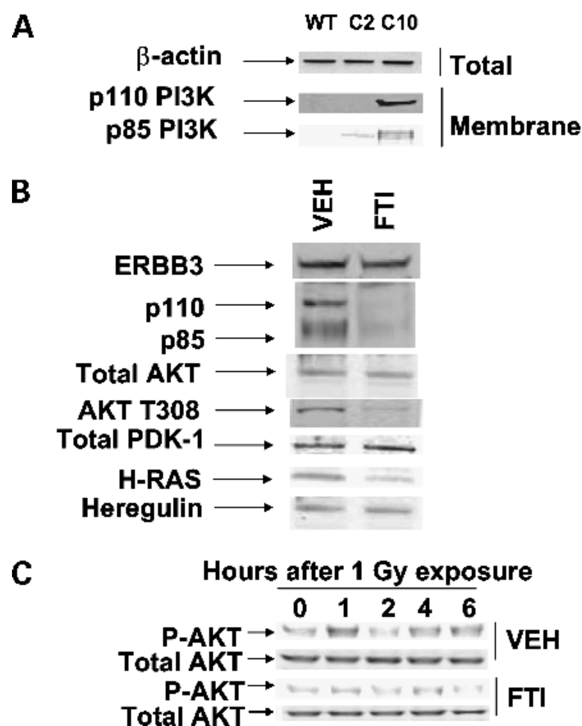

Figure 4. AKT activation and radiation resistance is dependent on H-RAS V12 – dependent membrane localization of PI3K. **A,** parental (WT), mutant K-RAS D13-deleted (C2), and H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were then either lysed to determine total β-actin expression or lysed with hypotonic buffer prior to preparation of plasma membranes, as described in Materials and Methods. Equal amounts of membrane protein were loaded onto SDS-PAGE and immunoblotting performed against the indicated membrane-associated proteins. Representative experiment ($n = 4$). **B,** H-RAS V12 (C10) cells were serum starved for 24 h in the presence or absence of the FTI277 (2 μmol/L) or vehicle (DMSO). Cells were then lysed with hypotonic buffer prior to preparation of membranes, as described above and in Materials and Methods. Equal amounts of membrane protein were loaded onto SDS-PAGE. Representative experiment ($n = 3$). **C,** H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h in the presence or absence of the FTI277 (2 μmol/L). Cells were irradiated (1 Gy) or mock exposed and homogenates taken 0 to 6 h afterwards to determine AKT S473 phosphorylation and total AKT expression, as described in Materials and Methods. Data shown are from 1, 2, 4, and 6 h after irradiation. Representative experiment ($n = 3$).

attachment of a farnesyl moiety (7, 11). Incubation of H-RAS V12 cells with an FTI (FTI277, 2 μmol/L for 24 hours) almost abolished the detection of H-RAS and PI3K (p110 and p85) in isolated plasma membranes (Fig. 4B). FTI277 treatment did not alter the total amount of membrane associated AKT, but reduced AKT phosphorylation and radiation-induced AKT phosphorylation, arguing that PI3K/PDK-1 activity had been lowered (Fig. 4C, data not shown). In general agreement with our findings elsewhere using the PI3K inhibitor LY294002, the AKT inhibitor perifosine and dominant-negative AKT, exposure of cells to FTI277 inhibited radiation-induced activation of AKT and radiosensitized H-RAS V12 cells (Fig. 4C; Table 2).

Inhibition of PDK-1 and *Src* Tyrosine Kinases Radiosensitizes H-RAS V12 Cells

We next examined whether modulation of PDK-1 function in HCT116 cell lines altered cell viability and radiosensitivity. The membrane localization of PDK-1 was investigated in parental (WT), K-RAS D13-deleted (C2), and H-RAS V12 (C10) cells (Fig. 5A). PDK-1 was present in membranes from both parental and H-RAS V12 cells but not in cells lacking expression of a mutated active RAS protein. As noted previously, PI3K and AKT were only present in membranes from H-RAS V12 cells. Treatment of H-RAS V12 and parental cells with an FTI for 24 hours reduced membrane association of H-RAS and PI3K p85 subunit but did not alter membrane association of PDK-1 and AKT (Fig. 5A, data not shown). The FTI-induced loss of membrane associated H-RAS and PI3K correlated with reduced AKT T308 phosphorylation, but surprisingly, did not significantly lower the phosphorylation of PDK-1 at positions of its proposed regulatory phosphorylation, including S241 and Y373/376.

Recently, an inhibitor of PDK-1, OSU-03012, with its chemical structure based on cyclooxygenase 2 inhibitors was reported (ref. 37; Fig. 2B). Tyrosine phosphorylation of PDK-1 at Y373/376 has been proposed to be mediated by *Src* family nonreceptor tyrosine kinases (38). Hence, further studies next determined whether small molecule inhibition of PDK-1 and *Src* family kinases altered the survival properties of HCT116 cells. OSU-03012 and PP2 inhibited the phosphorylation of AKT and PDK-1 in HCT116 cells (Fig. 5B and C). Treatment of H-RAS V12 cells with PP2 also reduced basal and radiation-stimulated tyrosine phosphorylation of PDK-1 at Y373/376 (Fig. 5C); the expression and Y416 phosphorylation of Src family nonreceptor tyrosine kinases did not seem to be significantly different in the HCT116 cell lines used in our studies (Fig. 5D). Incubation of H-RAS V12 cells with increasing concentrations of the PDK-1 inhibitor OSU-03012 or the *Src* inhibitor PP2 profoundly reduced plating efficiency and enhanced the lethal effects of radiation (Fig. 5E and F). In contrast, the effect of OSU-03012 and PP2 on the plating efficiency and radiosensitivity of parental HCT116 cells expressing K-RAS D13 was more modest, with no drug-induced alterations in the survival of parental cells after radiation exposure (Fig. 5G and H, data not shown).

Table 2. Treatment of H-RAS V12 (C10) cells with a FTI reduces plating efficiency and enhances radiosensitivity

Radiation dose (Gy)	Vehicle (DMSO) treatment	FTI (2 μmol/L) treatment
0	1.00 ± 0.08	0.52 ± 0.07*
1	0.88 ± 0.07	0.21 ± 0.04†
4	0.16 ± 0.04	0.02 ± 0.01†

NOTE: H-RAS V12 (C10) cells were plated in parallel to those described in Fig. 4 legend and serum starved for 24 hours with simultaneous exposure to a FTI (FTI277, 2 μmol/L). Cells were subjected to a 1- or 4-Gy exposure or to a mock exposure, as described in Materials and Methods. Colonies counted 10 to 14 days later as described in Materials and Methods. The numbers of colonies were expressed as percentage of the respective mock exposed group (defined as 1.00). Data are the means of six separate dishes from three separate experiments ± SE.

*$P < 0.05$ plating efficiency less than corresponding vehicle-treated cell value.

†$P < 0.05$ more radiosensitive than corresponding vehicle-treated value.

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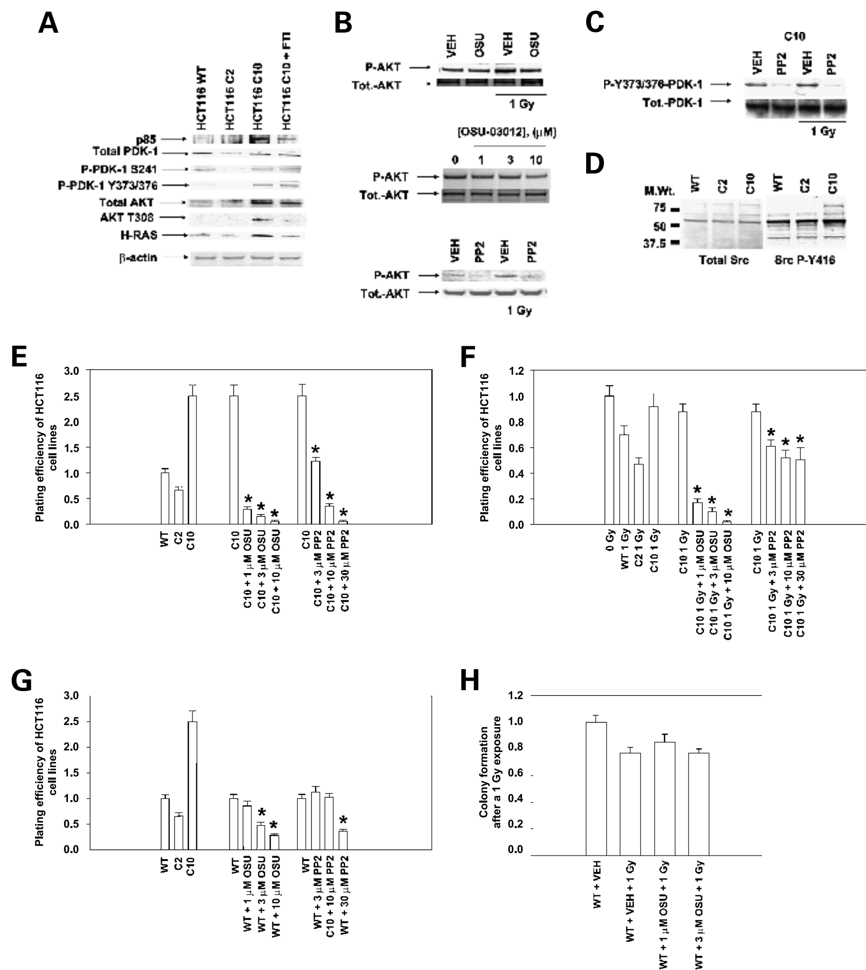

Figure 5. Membrane association of PDK-1 is mutated active RAS dependent, whereas membrane association of AKT and PI3K is H-RAS V12 dependent. **A,** parental (WT), mutant K-RAS D13-deleted (C2), and H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. For H-RAS V12 (C10) cells, cells were plated in parallel and serum starved for 24 h in the presence or absence of the FTI277 (2 μmol/L). Cells were then either lysed to determine total β-actin expression or lysed with hypotonic buffer before preparation of plasma membranes, as described in Materials and Methods. Equal amounts of membrane protein were loaded onto SDS-PAGE and immunoblotting performed against the indicated membrane-associated proteins. Representative experiment ($n = 4$). **B,** *top,* H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were treated with either vehicle (DMSO) or OSU-03012 (OSU, 1 μmol/L) 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and subjected to SDS-PAGE and immunoblotting to determine AKT S473 phosphorylation, as described in Materials and Methods. *Middle,* H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were treated with either vehicle (DMSO) or OSU-03012 (OSU, 0 – 10 μmol/L) 30 min before mock exposure. Cells were isolated 6 h after mock exposure and subjected to SDS-PAGE and immunoblotting to determine AKT S473 phosphorylation, as described in Materials and Methods. *Bottom,* H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were treated with either vehicle (DMSO) or PP2 (3 μmol/L) 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and subjected to SDS-PAGE and immunoblotting to determine AKT S473 phosphorylation, as described in Materials and Methods. **C,** H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were treated with either vehicle (DMSO) or PP2 (3 μmol/L) 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and subjected to SDS-PAGE and immunoblotting to determine PDK-1 Y373/376 phosphorylation, as described in Materials and Methods. **D,** parental (WT), mutant K-RAS D13-deleted (C2), and H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were isolated and subjected to SDS-PAGE and immunoblotting to determine Total *Src* family nonreceptor tyrosine kinase protein expression and Total *Src* family Y416 phosphorylation, as described in Materials and Methods. **E,** plating efficiency of parental (WT), mutant K-RAS D13-deleted cells (C2), or in H-RAS V12 cells (C10) was determined as described in the Materials and Methods. For drug treatments, HCT116 cells expressing H-RAS V12 (C10 cells) were serum starved for 24 h before radiation exposure (1 Gy) and treated 30 min before exposure with vehicle, OSU-03012 (*OSU*; 1, 3, or 10 μmol/L) or PP2 (3, 10, or 30 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. **F,** numbers of colonies were expressed as a fraction of the respective mock irradiated cells. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value. **G,** plating efficiency of parental (WT), mutant K-RAS D13-deleted cells (C2), and H-RAS V12 cells (C10) was determined as described in Materials and Methods. For drug treatments, parental (WT) HCT116 cells were serum starved for 24 h before radiation exposure (1 Gy) and treated 30 min before exposure with vehicle, OSU-03012 (*OSU*; 1, 3, or 10 μmol/L) or PP2 (3, 10, or 30 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value. **H,** for drug treatments, parental (WT) HCT116 cells were serum starved for 24 h before radiation exposure (1 Gy) and treated 30 min before exposure with vehicle, OSU-03012 (*OSU*; 1, 3, or 10 μmol/L). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. *Columns,* means of six separate dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding vehicle-treated cell value.

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To confirm that modulation of PDK-1 function by molecular methods promotes radiosensitization, H-RAS V12 and parental cells were transiently transfected with small molecule inhibitory RNA oligonucleotides against PDK-1 and irradiated (Fig. 6A, data not shown). In contrast to the cytotoxic effects observed using OSU-03012 as a single agent, treatment of HCT116 cells with inhibitory RNA molecules against PDK-1 did not significantly reduce plating efficiency in either H-RAS V12 or parental cells (Fig. 6B). However, reduced PDK-1 expression significantly enhanced radiosensitivity in H-RAS V12 cells, but not in parental HCT116 cells, in general agreement with findings using PI3K inhibitors and the AKT inhibitor perifosine (Fig. 6C and D).

Inhibition of Caspase-8 and Caspase-9 Blunts the Lethality and Radiosensitizing Properties of OSU-03012, but not Perifosine, Using Apoptosis Assays in H-RAS V12 Cells

We next examined the mechanisms by which OSU-03012 and perifosine modulated H-RAS V12 (C10) cell viability and radiosensitivity in apoptosis assays 96 hours after exposure. OSU-03012 and perifosine enhanced cell morbidity in H-RAS V12 cells after drug addition (Fig. 7A). However, whereas OSU-03012 also enhanced the lethality of radiation in H-RAS V12 (C10) cells, in general agreement with our findings in colony formation experiments, no further increase in radiation toxicity was observed using perifosine which was in contrast to our findings using long-term clonogenic assays.

Three major pathways/mechanisms for the induction of apoptosis have been described by many laboratories in the literature; the extrinsic caspase-8/caspase-10–dependent death receptor pathway, the intrinsic caspase-9–dependent mitochondrial pathway and an ER stress pathway which can cause mitochondrial dysfunction via caspase-2 leading to a caspase-9–dependent induction of cell death (39–42). Death receptor signaling by caspase-8 in HCT116 cells has been shown by other groups to proceed by causing mitochondrial dysfunction and activation of pro-caspase-9 (43).

Inhibition of caspase-8, caspase-9, or both inhibitors combined did not alter the cytotoxic properties of perifosine (Fig. 7B). Inhibition of either caspase-8 or caspase-9 significantly reduced the lethality of OSU-03012 (Fig. 7C). Inhibition of either caspase-9 or to a greater extent caspase-8 reduced, but did not abolish, the radiosensitizing properties of OSU-03012. Combined inhibition of caspase-8 and caspase-9 did not further blunt radiosensitization by OSU-03012 (data no shown). Collectively, these findings suggest that proposed PDK-1 inhibitor OSU-03012 promotes radiosensitization via multiple apoptotic pathways and does so in a manner which is dissimilar to the proposed AKT inhibitor perifosine.

Discussion

Several studies have linked mutated active forms of K-RAS and H-RAS to enhanced radiation resistance in a variety of unrelated human tumor cell types or in rodent fibroblasts. The present studies were initiated to examine the radioprotective properties of a mutant active K-RAS protein and a mutant active H-RAS protein in an isogenic epithelial carcinoma cell system, and the effect inhibitors of the PI3K/PDK-1/AKT pathway had upon radiosensitivity. Loss of K-RAS D13 expression in HCT116 cells reduced basal activity of ERK1/2, AKT and JNK1/2 as well as almost

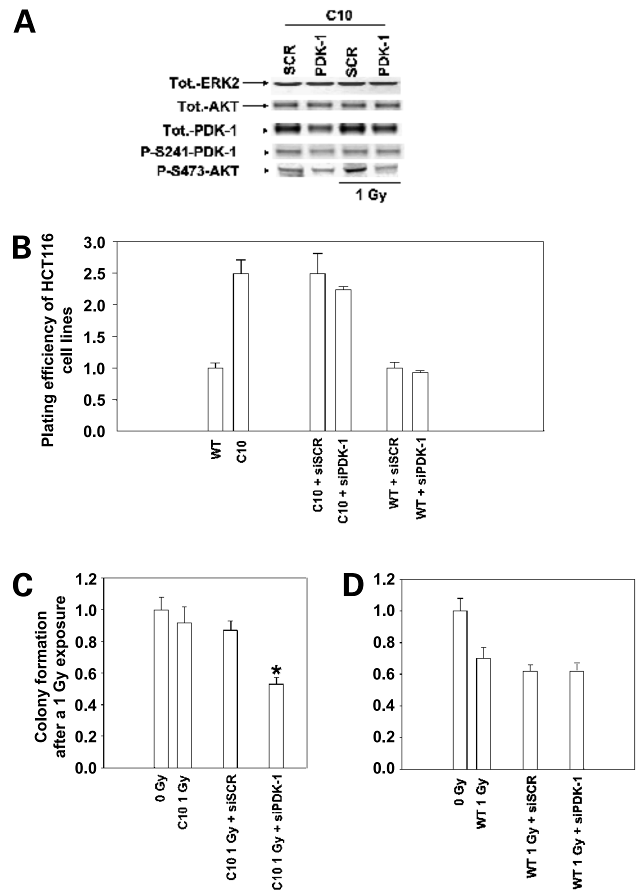

Figure 6. siRNA-mediated inhibition of PDK-1 expression radiosensitizes H-RAS V12 (C10) cells but not parental (WT) HCT116 cells. **A,** H-RAS V12 (C10) cells were transfected with either a siRNA molecule against PDK-1, a scrambled PDK-1 siRNA, or a sense PDK-1 siRNA (data not shown) as described in Materials and Methods. Twenty-four hours after replating, cells were serum starved for 24 h and then irradiated (1 Gy). Six hours after irradiation, cells were processed for immunoblotting to determine the expression and phosphorylation of various proteins. Representative experiment ($n = 3$). **B, C, D,** and **E,** H-RAS V12 (C10) cells and parental (WT) cells were transfected with either a siRNA molecule against PDK-1 (siPDK-1), a scrambled PDK-1 siRNA (siSCR) as described in Materials and Methods. Forty-eight hours after transfection, cells were replated for colony formation assays and 24 h after plating for these assays, serum starved for 24 h, and then irradiated (1 Gy). Media containing serum was added 24 h after exposure, and 10 to 14 d later colonies were counted as indicated in Materials and Methods. *Columns,* means of six separate transfection dishes per experiment from two separate experiments; *bars,* ±SE. *, $P < 0.05$ less than corresponding siSCR value. **B,** siPDK-1 did not alter plating efficiency in HCT116 cell lines. **C, D,** and **E,** siPDK-1 radiosensitizes H-RAS V12 cells but not parental HCT116 cells.

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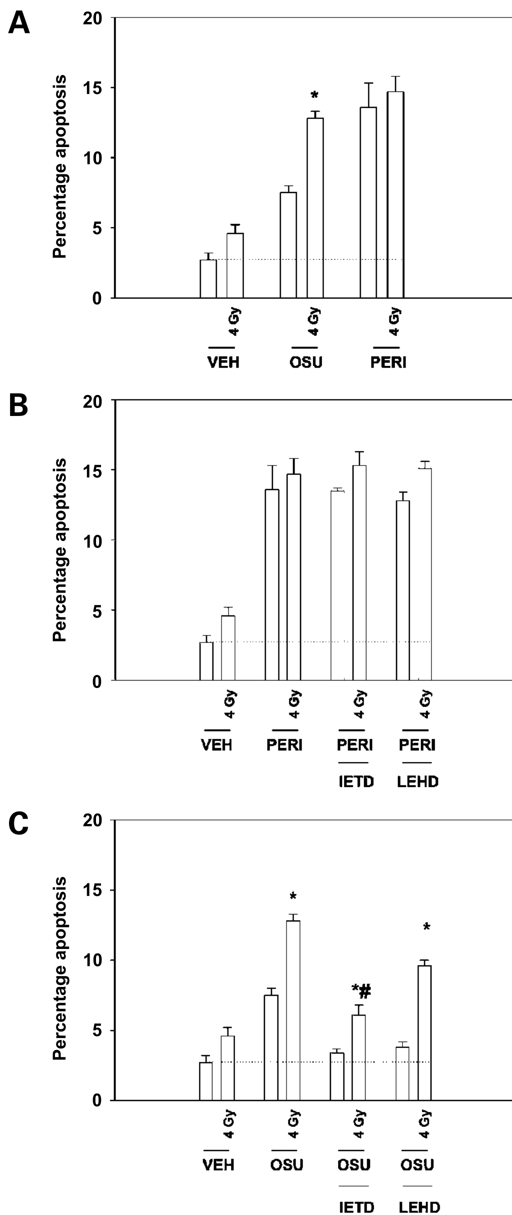

Figure 7. OSU-03012 promotes cell killing and radiosensitization via caspase-8-dependent and caspase-9-dependent pathways. H-RAS V12 (C10) cells were plated in parallel and serum starved for 24 h. Cells were treated with either vehicle (*VEH*, DMSO), OSU-03012 (*OSU*, 1 μmol/L), or perifosine (*PERI*, 1 μmol/L) 30 min before irradiation (4 Gy). For studies using caspase inhibitors, cells were treated with vehicle (DMSO), with the caspase-8 inhibitor IETD (50 μmol/L) or the caspase-9 inhibitor LEHD (50 μmol/L) 30 min before irradiation. Cells were isolated 96 h after irradiation, fixed to glass slides, and stained as described in Materials and Methods. Morphologic assessment of apoptosis was determined by two operators blinded to the treatment condition. **A,** OSU-03012 but not perifosine enhances the lethality of radiation in apoptosis assays. **B,** lethality of perifosine is not enhanced by radiation and is not reduced by inhibition of either caspase-8 or of caspase-9 in apoptosis assays. **C,** inhibition of either caspase-8 or caspase-9 blunts but does not abolish radiosensitization of H-RAS V12 (C10) cells by OSU-03012 in apoptosis assays. *Columns,* means of six independent slides from two separate experiments; *bars,* ±SE. *, $P < 0.05$ greater than corresponding vehicle-treated value; #, $P > 0.05$ for value compared to corresponding irradiated value.

abolishing radiation-induced activation of AKT. Introduction of H-RAS V12 into these cells restored basal ERK1/2 and AKT activity, but not that of JNK1/2, to those levels found in WT cells, and potentiated AKT activation by radiation. In contrast to studies in rodent fibroblasts transfected to express activated K-RAS and H-RAS proteins, basal or stimulated p38 activity was not detected in HCT116 cells. These findings argue that in HCT116 carcinoma cells, H-RAS V12 preferentially enhances activation of the PI3K/AKT pathway.

The effect of PI3K/PDK-1/AKT pathway inhibitors on cell survival was investigated in the HCT116 cell lines expressing K-RAS D13 and H-RAS V12; these were the cell lines exhibited the highest basal and radiation-stimulated AKT activity profiles. Inhibition of basal or the modest levels of radiation-stimulated PI3K/AKT activity in parental cells expressing K-RAS D13 did not significantly alter cell survival. In contrast, inhibition of PI3K or expression of dominant-negative AKT enhanced the lethality of radiation in cells expressing H-RAS V12. These findings correlated well with the strong prolonged activation of AKT by radiation in cells expressing H-RAS V12 compared with the weaker activation of AKT observed in parental cells. Additional studies then examined a range of novel AKT PH domain inhibitors in their abilities to modulate cell survival of cells expressing H-RAS V12. Eight AKT PH domain inhibitors were tested [perifosine, SH-(5, 23-25), and ml-(14-16); all inhibitors caused a dose-dependent reduction in the plating efficiency of H-RAS V12 cells in colony formation assays and blocked stimulated AKT activity, but only perifosine reduced basal and stimulated AKT activity, and enhanced radiosensitivity, but only radiosensitivity as measured in long-term colony formation assays. This data suggests that perifosine is a relatively weak radiosensitizer.

The abilities of the *SH* series of AKT inhibitors to alter cell survival were recently examined in lung carcinoma cells. In the lung cancer cells, it was noted that in the 1 to 10 μmol/L dose range, the AKT PH domain inhibitors only maintained inhibition of AKT function for ~18 hours after drug exposure (36). Perifosine but not SH-5 maintained inhibition of basal AKT activity for at least 24 hours. Previous studies by this laboratory examining the interactions of mitogen-activated protein kinase kinase 1/2 inhibitors with ionizing radiation have argued that prolonged >60 hours of inhibition of the ERK1/2 pathway is required to radiosensitize tumor cells and that a ~24 to 48 hours of inhibition of ERK1/2 signaling has little effect on long-term cell survival even when effects on plating efficiency were evident (25, 39). One explanation for our findings is that as ionizing radiation is believed to predominantly cause post-mitotic cell death, taking several generations to become manifest, rather than a rapid apoptosis response, it is possible that a prolonged (intense) suppression of survival signaling by kinases is required to observe significant radiosensitizing effects.

Our data examining the abilities of SH-/ml- AKT inhibitors, perifosine and, OSU-03012 to modulate cell

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survival and in parallel inhibit basal/stimulated AKT phosphorylation were in poor agreement; notwithstanding our findings that dominant-negative AKT and a siRNA against PDK-1 blocked AKT signaling and enhanced radiosensitivity. That is, the degree to which molecular tools and therapeutic drugs modulated AKT activity and plating efficiency/radiosensitivity did not correlate. In addition, whereas inhibition of caspase-8 and caspase-9 both reduced OSU-03012 toxicity, neither altered cell killing by perifosine: a priori based on absolute drug specificity it would have been predicted that their modes of cell killing would have been similar. Collectively, these findings suggest that although loss of PDK-1/AKT function radiosensitizes HCT116 cells expressing H-RAS V12, neither perifosine nor OSU-03012 are specific inhibitors of the PI3K/PDK-1/AKT pathway.

One possible reason for our observation that radiation caused a pronounced activation of AKT in H-RAS V12 cells is that H-RAS V12 has been reported to preferentially bind to the p110 subunit of PI3K and cause its partial activation (40). Thus, H-RAS V12 may act as a binding protein to localize PI3K p110 in the plasma membrane which facilitates PI3K activation, as was previously noted for Raf-1 (30, 31). We determined that a FTI abolished membrane association of H-RAS and PI3K in H-RAS V12 cells, which also abolished radiation-induced AKT activation. Furthermore, and in contrast to the PI3K translocating properties of H-RAS V12, K-RAS D13 did not promote PI3K localization in the plasma membrane of WT HCT116 cells. Instead, K-RAS D13 promoted Raf-1 membrane localization. Collectively, these findings show that activated forms of H-RAS and K-RAS differentially act to membrane-localize PI3K and Raf-1 in HCT116 carcinoma cells.

In cells lacking expression of a mutated active RAS protein, neither PI3K, PDK-1 nor AKT were membrane localized. Expression of H-RAS V12 in cells lacking expression of a mutated active RAS protein promoted membrane association of PI3K, PDK-1 and AKT. In H-RAS V12 cells, we discovered, however, that a 24-hour FTI exposure, which reduced membrane-associated levels of H-RAS V12 and PI3K, did not alter the membrane association of either PDK-1 or AKT. In contrast to findings with H-RAS V12, PI3K, and AKT were not present in membranes from cells expressing K-RAS D13, whereas PDK-1 was membrane localized in cells expressing K-RAS D13 or H-RAS V12. Thus, membrane association of PDK-1 was activated RAS, dependent in HCT116 cells, and our data suggests that the rate at which PDK-1 dissociated from membranes seemed to be relatively slow. The tyrosine phosphorylation status of membrane associated PDK-1 was noted to be different in cells expressing H-RAS V12 and K-RAS D13, with PDK-1 Y373/376 phosphorylation being elevated in cells expressing H-RAS V12. The inhibitor of *Src* family nonreceptor tyrosine kinases PP2 abolished PDK-1 tyrosine phosphorylation and AKT activity in H-RAS V12 cells, whereas PP2 did not significantly alter AKT phosphorylation in parental cells.[7] This suggested that *Src* family proteins are either expressed at a greater level in H-RAS V12 cells or are more active when this oncogene is expressed. However, in studies examining *Src* family protein expression in parental and H-RAS V12 cells, and Y416 and Y527 phosphorylation of these proteins, no clear demonstration could be found that the expression or phosphorylation of any *Src* family protein was significantly altered. Additional studies will be required to determine more definitively whether tyrosine phosphorylation of PDK-1 in H-RAS V12 cells is mediated by a *Src* family protein kinase or whether tyrosine phosphorylation of PDK-1 is mediated by a related membrane associated tyrosine kinase that is inhibited by PP2.

Based on the findings described above, the ability of a novel PDK-1 inhibitor (OSU-03012) and PP2 to alter HCT116 cell survival was determined. Neither OSU-03012 nor PP2 altered the radiosensitivity of parental HCT116 cells, and both drugs weakly modulated parental HCT116 cell plating efficiency. However, in HCT116 cells expressing H-RAS V12, both OSU-03012 and PP2 significantly reduced both plating efficiency and radiosensitivity. The relevance of PDK-1 in modulating the radiosensitivity of H-RAS V12 cells was confirmed using small inhibitory RNA molecules against PDK-1. Thus, membrane association of PDK-1 per se does not correlate with PDK-1 playing a central protective role in the survival processes of cells following irradiation. Our data also argues that PDK-1 Y373/376 phosphorylation but not S241 phosphorylation is indicative of a potential role for PDK-1 in tumor cell radioresistance.

Basal AKT activity, as judged by phosphorylation of T308 and S473, was similar in both parental HCT116 cells and in HCT116 cells expressing H-RAS V12. However, radiation more potently activated AKT in H-RAS V12 cells than in parental cells and inhibition of the PI3K/PDK-1/AKT pathway in H-RAS V12 cells, but not parental cells, enhanced the toxicity of radiation. This data, findings with the AKT inhibitors perifosine and SH-5 notwithstanding, suggests that basal levels of AKT activity/phosphorylation in resting cells may not be a good predictor of whether a tumor cell will respond to inhibition of the PI3K/PDK-1/AKT pathway, particularly when a therapeutic inhibitor is combined with a toxic agent such as ionizing radiation. In contrast, it seems as though PDK-1 tyrosine phosphorylation and the degree to which AKT becomes activated after irradiation correlate more closely with the effect on cell survival of PI3K, PDK-1, and AKT inhibitors.

Recent studies by other groups have suggested that cell lines with higher basal AKT activities are more sensitive to the toxic effects of AKT and PDK-1 inhibitors than those with lower levels of AKT and PDK-1 activity (36, 37). Hence, our data potentially adds two additional levels of complexity to this concept: (1) cells that strongly

[7] R. Carón and P. Dent, unpublished observations.

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activate the PI3K/PDK-1/AKT pathway after irradiation, or that have a higher potential to activate the pathway due to H-RAS V12–dependent translocation of PI3K to the plasma membrane, are likely to be more sensitive to the toxic effects of PI3K/PDK-1/AKT inhibitors; and (2) inhibition of AKT signaling for a short period of time reduces cell viability but does not necessarily alter radiosensitivity. If one simplistically uses electrical circuits as analogous to signal transduction pathways in cells then the ''current'' within a pathway (e.g., AKT basal activity) only partially defines the importance of that pathway in maintaining cell viability. The ''potential difference'' of a pathway, defined as the activation of upstream molecules (e.g., PI3K) by oncogenes such as H-RAS V12 and by external stimuli such as radiation, also can define how important a given pathway is to promoting cell survival. The overall ''power'' of the pathway to influence cell viability is thus the product of both the basal pathway activity and the potential for this pathway to be activated by toxic stimuli. Additional studies will be required to validate this hypothesis in other isogenic tumor cell lines.

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