Targeting of AKT1 enhances radiation toxicity of human tumor cells by inhibiting DNA-PKcs-dependent DNA double-strand break repair

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Abstract

We have already reported that epidermal growth factor receptor/phosphatidylinositol 3-kinase/AKT signaling is an important pathway in regulating radiation sensitivity and DNA double-strand break (DNA-dsb) repair of human tumor cells. In the present study, we investigated the effect of AKT1 on DNA-dependent protein kinase catalytic subunit (DNA-PKcs) activity and DNA-dsb repair in irradiated non-small cell lung cancer cell lines A549 and H460. Treatment of cells with the specific AKT pathway inhibitor API-59CJ-OH (API; 1-5 μmol/L) reduced clonogenic survival between 40% and 85% and enhanced radiation sensitivity of both cell lines significantly. As indicated by fluorescence-activated cell sorting analysis (sub-G_1 cells) and poly(ADP-ribose) polymerase cleavage, API treatment or transfection with *AKT1*-small interfering RNA (siRNA) induced apoptosis of H460 but not of A549 cells. However, in either apoptosis-resistant A549 or apoptosis-sensitive H460 cells, API and/or *AKT1*-siRNA did not enhance poly(ADP-ribose) polymerase cleavage and apoptosis following irradiation. Pretreatment of cells with API or transfection with *AKT1*-siRNA strongly inhibited radiation-induced phosphorylation of DNA-PKcs at T2609 and S2056 as well as repair of DNA-dsb as measured by the γ-H2AX foci assay. Coimmunoprecipitation experiments showed a complex formation of activated AKT and DNA-PKcs, supporting the assumption that AKT plays an important regulatory role in the activation of DNA-PKcs in irradiated cells. Thus, targeting of AKT enhances radiation sensitivity of lung cancer cell lines A549 and H460 most likely through specific inhibition of DNA-PKcs-dependent DNA-dsb repair but not through enhancement of radiation-induced apoptosis. [Mol Cancer Ther 2008;7(7):1772–81]

Introduction

In patients with non-small cell lung cancer (NSCLC), chemotherapy offers only a small improvement and cell lines derived from these tumors exhibit an intrinsic resistance to both chemotherapy and radiotherapy *in vitro* compared with other types of cancer cells (1). Therefore, it is necessary to explore new strategies to improve treatment efficiency, especially with respect to a curative approach in radiotherapy (2, 3). One factor known to increase tumor cell resistance to radiation is overexpression or mutationally activated oncogenes such as epidermal growth factor receptor (EGFR) and RAS (4, 5). Mutations in RAS, most frequently in K-RAS, are seen in 30% of all lung adenocarcinomas. Similarly, 40% of NSCLC present an amplification of EGFR (6). EGFR activation results in stimulation of the phosphatidylinositol 3-kinase (PI3K)/AKT survival pathway involved in resistance of NSCLC to radiotherapy/chemotherapy (7, 8). Thus, the importance of PI3K/AKT targeting for overcoming resistance of tumors to radiotherapy has been tested *in vitro* and *in vivo* and the results indicate this cascade as promising target (9–11). However, the toxicity of most inhibitors applied (e.g., PI3K inhibitor LY294002) does not allow clinical use. Therefore, the next generation of inhibitors of PI3K or specific targeting of kinases downstream of PI3K, such as AKT/protein kinase B, could be an applicable alternative strategy.

The serine/threonine kinase AKT/protein kinase B is expressed in three isoforms: AKT1/protein kinase Bα, AKT2/protein kinase Bβ, and AKT3/protein kinase Bγ. AKT kinase is efficiently induced by growth factors, like EGFR ligands, or by ionizing radiation (IR) through the EGFR-mediated activation of PI3K (8, 12–17). Activation of AKT via PI3K occurs through phosphorylation by PDK-1 and/or PDK-2 at serine and threonine residues (Thr^{308} and Ser^{472}/Ser^{473}). Especially, AKT phosphorylation at these residues is associated with resistance to chemotherapy/radiotherapy (18–20) and it has been proposed that activated AKT promotes survival of cells exposed to IR through inhibition

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of apoptosis (7). Apoptosis induced by DNA damage is typically associated with activation of a family of proteases, the caspases, as a result of a sequence of mitochondria-mediated events (21). The antiapoptotic activity of AKT is mediated through AKT-dependent phosphorylation and thus inactivation of proapoptotic Bad and caspase-9 proteins (22).

Previously, we have reported that targeting of EGFR and PI3K signaling in various cultured tumor cell lines from human NSCLC, head and neck, and breast cancers impairs repair of radiation-induced DNA double-strand breaks (DNA-dsb) resulting in enhanced radiosensitivity selectively of cell lines presenting a mutated K-RAS gene (14, 15). DNA-dsb are the most important DNA lesions leading to cell kill following exposure to IR (23). Two processes are primarily involved in the repair of DNA-dsb, that is, nonhomologous end-joining (NHEJ) and homologous recombination (24), but NHEJ is the predominant process of DNA-dsb repair. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is the key enzyme of NHEJ repair. Activation of DNA-PKcs requires the phosphorylation of specific amino acid residues in its catalytic subunit, among which T2609 and S2056 have been identified to be essential for efficient rejoining of DNA-dsb during NHEJ (25). Likewise, deletion of these phosphorylation sites results in enhanced cellular sensitivity to IR (26–29).

Based on our previously published results indicating a link among EGFR/PI3K/AKT signaling, activation of DNA-PKcs, and NHEJ repair, in the current study, we investigated the possible role of AKT as an essential regulatory component of DNA-PKcs and DNA-dsb repair in irradiated cells. Evidence will be provided that AKT activity is essential for radiation-induced phosphorylation of DNA-PKcs at T2609 and S2056. Consequently, AKT targeting enhances radiation sensitivity of NSCLC tumor cells through inhibition of NHEJ repair.

Materials and Methods

Antibodies and Inhibitors

P-DNA-PKcs (T2609) and P-H2AX (S139) were purchased from Biomol. P-DNA-PKcs (S-2056) was kindly provided by Prof. Dr. David Chen (Division of Molecular Radiation Biology, University of Texas Southwestern Medical Center). DNA-PKcs, AKT1, and P-AKT (Ser^{472}/Ser^{473}) antibodies were products of BD Biosciences. Cleaved poly(ADP-ribose) polymerase (PARP) and P-AKT (Thr^{308}) were purchased from Cell Signaling Technology. Vectashield mounting medium with 4′,6-diamidino-2-phenylindole was prepared from Linaris. The AKT pathway inhibitor API-59CJ-OH (API) is a derivate of API-59-OMe as already described (30). EGF, transforming growth factor-α, amphiregulin, and anti-actin antibody were received from Sigma-Aldrich. Caspase inhibitor z-VAD.fmk was a product of R&D Systems. Small interfering RNA (siRNA) against *AKT1*, *DNA-PKcs*, and control siRNA were prepared by Dharmacon. Lipofectamine 2000 and Opti-MEM were purchased from Invitrogen.

Cell Lines

Established human lung adenocarcinoma cell lines A549 and H460 were used. Cells were cultured in DMEM (A549) or RPMI 1640 (H460) routinely supplemented with 10% FCS and 1% penicillin-streptomycin and incubated in a humidified atmosphere of 93% air/7% CO_2 at 37°C.

Clonogenic Assay

Clonogenic cell survival of cells pretreated with the AKT pathway inhibitor alone or in combination with IR was analyzed by means of standard colony formation assay. To investigate antiproliferative and radiosensitizing effects of API, confluent cell cultures pretreated for 72 h with the inhibitor were trypsinized, plated at a constant cell density (250 cells per well in six-well plate), and incubated for 10 days to allow for colony growth. In case of radiosensitivity measurements, cells were X-ray irradiated 24 h after plating. After 10 days of incubation, cultures were fixed and stained. Colonies formed with more than 50 cells were scored as survivors. Clonogenic fraction of irradiated cells was normalized to the plating efficiency of unirradiated controls.

Flow Cytometric Analysis of Apoptosis

Cells were seeded 24 h before treatment with API (1 μmol/L) or transfection with AKT1-siRNA (50 nmol/L). Control cells were treated with DMSO or transfected with control-siRNA. After API treatment for 72 h and 4 days after siRNA transfection, cultures were mock irradiated or irradiated with 5 Gy. API-treated cells were trypsinized at 24 and 72 h after irradiation (that is, 4 and 6 days after API treatment). In siRNA-transfected conditions, cells were collected 72 h after irradiation (7 days after siRNA transfection). Floating cells in culture medium were included to each condition as well. Cells were fixed with 70% ethanol and stored overnight at -20°C. For analysis, cells were collected by centrifugation, incubated for 10 min with RNase (100 μg/mL) in PBS/1% bovine serum albumin, and resuspended in 1 mL PBS with propidium iodide (10 μg/mL) for analysis. Cell number in sub-G_1 region of the cell cycle was determined and calculated as a percentage of the total cell population. Mean ± SEM of at least three independent experiments were calculated and graphed.

γ-H2AX Foci Assay

γ-H2AX foci assay was applied for determination of residual DNA-dsb. Therefore, cells were cultured on glass slides and 24 h later were either transfected with control and specific siRNA or treated with DMSO and API. Four days after siRNA transfection and 72 h after API treatment, cultures were irradiated with single dose of IR (1-5 Gy) and incubated at 37°C for 24 h. Thereafter, slides were prepared as reported previously. Cells (n = 120-150) were counted per condition (15). In an alternative experiment, effect of API on residual DNA-dsb was analyzed at different time points (that is, 1-24 h after irradiation dose of 2 Gy).

siRNA Transfection and Western Blotting Analysis

Transfection of A549 and H460 cells with siRNA against *AKT1* and *DNA-PKcs* (50 nmol/L; Dharmacon) as well as Western blotting were done as described previously (15).

Immunoprecipitation

Immunoprecipitation experiments using P-AKT and AKT1 antibodies were done as described previously (14). Coimmunoprecipitation of DNA-PKcs was tested by immunoblotting using P-DNA-PKcs (T2609) and DNA-PKcs antibodies.

Results

API Inhibits Proliferation and Enhances Radiation Sensitivity of K-RAS Mutated NSCLC Cell Lines A549 and H460

As reported previously (14, 15), inhibition of EGFR or PI3K enhances radiation sensitivity of tumor cells presenting a K-RAS mutation. Therefore, we investigated whether direct AKT targeting affects clonogenic survival of unirradiated and irradiated K-RAS_{mt} human tumor cells. As shown in Fig. 1A, a concentration-dependent reduction of clonogenic activity of the unirradiated cell lines was observed after API treatment. The combination of API treatment with single-dose irradiation (1-5 Gy) significantly enhanced radiation sensitivity of both cell lines in a dose-dependent manner (Fig. 1B). These data were used to calculate the dose D37, which is required to reduce survival to 37%. For A549 cells, D37 was 2.52 Gy, whereas the combination of irradiation and API treatment with concentrations of 1 and 2.5 μmol/L resulted in D37 values of 1.77 and 1.42 Gy, respectively. The resulting dose-modifying factors of API in A549 cells could be calculated to be 1.42 (1.0 μmol/L API) and 1.77 (2.5 μmol/L API). In H460 cells, D37 without API is 2.58 Gy. API at 1 and 2.5 μmol/L reduced this value to 2.16 Gy (1.0 μmol/L API) and 1.57 Gy (2.5 μmol/L API), respectively. Consequently, a reduction in D37 was apparent due to API treatment resulting in dose-modifying factors of 1.19 (1.0 μmol/L API) and 1.64 (2.5 μmol/L API).

Differential Effect of API on Radiation-Induced Apoptosis

It is generally believed that blockage of AKT activity stimulates radiation-induced apoptosis (9), which may result in an enhanced radiation toxicity. Thus, we investigated whether radiosensitization mediated through AKT targeting is the result of stimulated radiation-induced apoptosis. As shown by flow cytometry (Fig. 2A), the proportion of A549 sub-G_1 cells induced either by API (1 μmol/L) or by IR (5 Gy single dose) is below 1% and this fraction of apoptotic sub-G_1 A549 cells could not further be enhanced by combining radiation exposure with API treatment. Likewise, after transfection with AKT1-specific siRNA, radiation exposure (single dose of 5 Gy) did not significantly enhance the proportion of sub-G_1 A549 cells (Fig. 2B).

For H460 cells, API treatment as well as radiation exposure significantly enhanced the fraction of apoptotic sub-G_1 cells (API ~7-fold, 5 Gy ~2-fold). Combined treatment with API and IR did not result in a significant

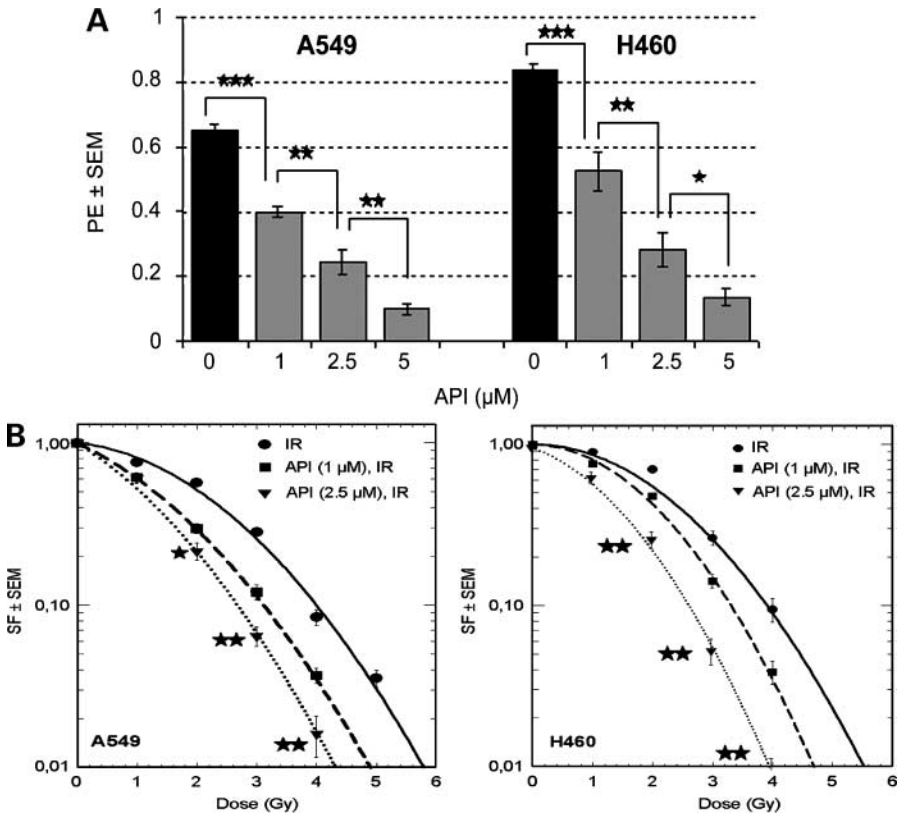

Figure 1. API inhibits proliferation and enhances radiation sensitivity of K-RAS mutated NSCLC cell lines A549 and H460. **A,** log-phase cells were pretreated with different concentrations of API for 72 h and plated for colony formation. Cultures were incubated to allow colony growth for 10 d. Thereafter, colonies were fixed and stained and the number of colonies with more than 50 cells was counted. Mean ± SEM plating efficiency of at least four parallel data sets from three independent experiments. ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$, statistically significant API dose-dependent reduction of plating efficiency (Student's *t* test). **B,** log-phase A549 and H460 cells were pretreated with 1 and 2.5 μmol/L API for 72 h and plated for colony formation. After 24 h, cultures were mock irradiated or irradiated with single doses (1-5 Gy) and incubated for colony formation within 10 d. Colonies formed were counted and survival fractions were calculated as described in Materials and Methods. Data points shown represent the mean surviving fraction of six parallel data sets from at least two independent experiments ($n = 12$); *bars,* SEM. API at 1 and 2.5 μmol/L induced radiosensitization after 2 Gy in both cell lines significantly ($P < 0.01$, Student's *t* test). **, $P < 0.01$; *, $P < 0.05$, significant increases in radiosensitization by API (comparing API concentrations of 1-2.5 μmol/L).

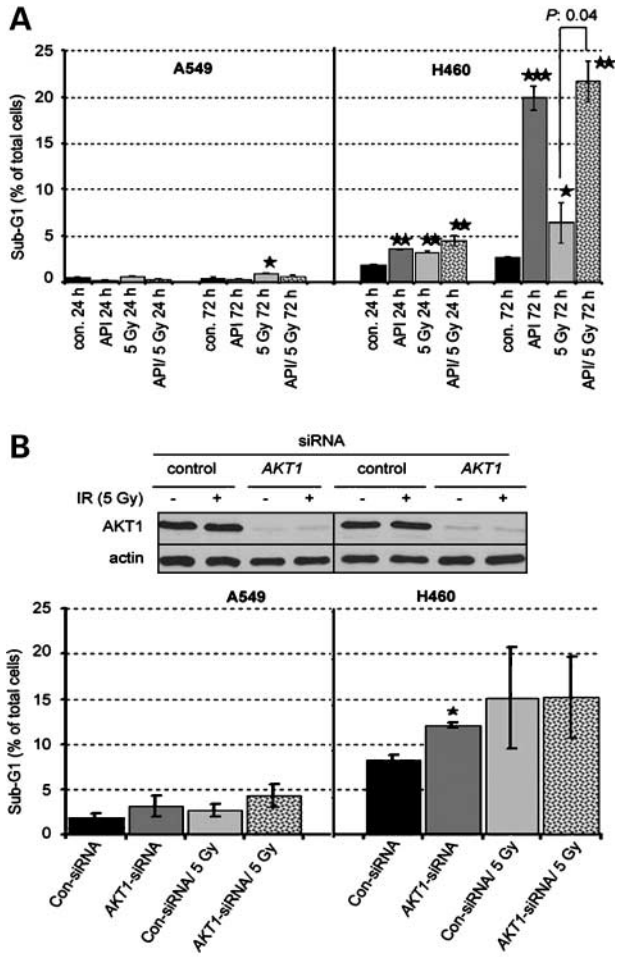

Figure 2. AKT targeting does not enhance radiation-dependent apoptosis of A549 and H460 cells. **A,** log-phase cells were treated with API (1 μmol/L) or DMSO for 72 h and mock irradiated or irradiated with 5 Gy. After irradiation for 24 and 72 h (4 or 6 d after API treatment), cells were collected and fluorescence-activated cell sorting analysis was done as described in Materials and Methods. Mean ± SEM of at least three independent experiments. ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$, significant enhancement of the sub-G_1 fraction of different treatment conditions compared with control (Student's *t* test). Except for the significant difference in sub-G_1 fraction between IR and API/IR at 72 h in H460 cells, the differences in sub-G_1 fraction between the treatment conditions (IR versus API/AR or API versus API/IR) were not statistically significant. **B,** cells were transfected with 50 nmol/L control-siRNA or *AKT1*-siRNA and were mock irradiated or irradiated with 5 Gy 4 d after transfection. Following irradiation, protein samples were prepared and the efficiency of *AKT1*-siRNA to repress AKT1 protein expression was analyzed by Western blotting. Actin was used as loading control. In a parallel experiment, cells were irradiated 4 d after transfection; 72 h after irradiation, cells were collected for fluorescence-activated cell sorting analysis. Percentage of sub-G_1 was determined as described in Materials and Methods. Mean ± SEM of three independent experiments. *, $P < 0.05$, significant enhancement of the sub-G_1 fraction following irradiation compared with control in H460 cells (Student's *t* test).

further increase of sub-G_1 H460 cells; under these conditions primarily, the API effect could be observed (Fig. 2A). Targeting of AKT1 in H460 cells by specific siRNA enhanced the sub-G_1 fraction in unirradiated cells from ~8% to ~12% and exposure to IR (single dose of 5 Gy) resulted in an apoptotic fraction of ~15% sub-G_1 cells. Combined AKT1-siRNA treatment and radiation exposure did not further stimulate the proportion of sub-G_1 H460 cells (Fig. 2B).

API-Induced Apoptosis in H460 Is through Caspase Activity and PARP Cleavage

In both cell lines tested, the proportion of apoptosis induction by different modalities (that is, API treatment as well as IR alone or the combination of both modalities) correlates to the level of PARP cleavage. The enhanced apoptotic sub-G_1 fraction in unirradiated H460 cells described above is reflected by a strong API-dependent induction of PARP cleavage (Fig. 3A). However, as expected from Fig. 2A, in unirradiated A549 cells, API treatment did not induce PARP cleavage (Fig. 3A). Interestingly, in contrast to IR-induced PARP cleavage in DMSO-treated cells, pretreatment with API resulted in an inhibition of radiation-induced cleavage. In agreement with these results as shown by densitometry values, API treatment in combination with irradiation led to less than additive effect on PARP cleavage in H460 cells (Fig. 3A). To investigate whether the apoptosis induction in H460 cells is sensitive to the caspase inhibitor z-VAD.fmk, the proportion of sub-G_1 cells in cultures exposed to API treatment and irradiation with or without z-VAD.fmk was

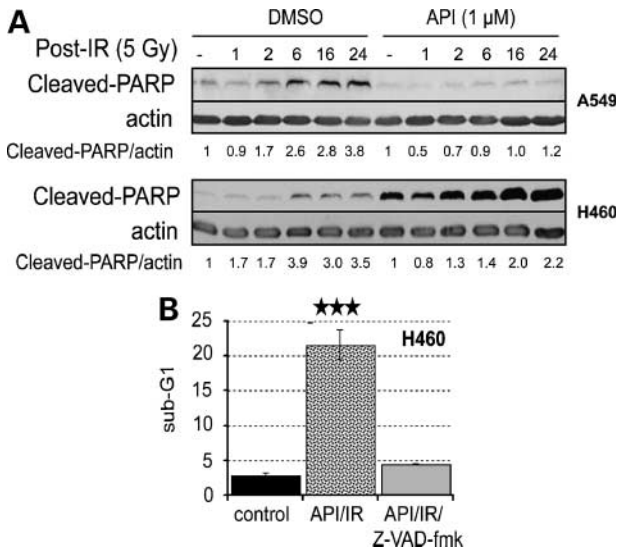

Figure 3. API-induced apoptosis in H460 depends on caspase activity and PARP cleavage. **A,** cells were pretreated with DMSO or API for 72 h and mock irradiated or irradiated with single dose of 5 Gy. At different times after irradiation (1-24 h), protein samples were prepared and subjected to SDS-PAGE. PARP cleavage (85 kDa) was detected by specific antibodies to cleavage products. Actin was used as loading control. Densitometry values represent the ratio of cleaved PARP/actin (normalized to 1 in unirradiated controls). **B,** H460 cells were pretreated with DMSO, API (1 μmol/L), or API (1 μmol/L)/z-VAD.fmk (100 μmol/L) for 3 d and mock irradiated or irradiated with 5 Gy. After irradiation for 72 h (6 d after API or API/z-VAD.fmk treatment), cells were collected and percentage of sub-G_1 cells was calculated for each condition as described earlier. *, $P < 0.0001$, significant difference in sub-G_1 fraction between control and API/IR treatment condition (Student's *t* test).

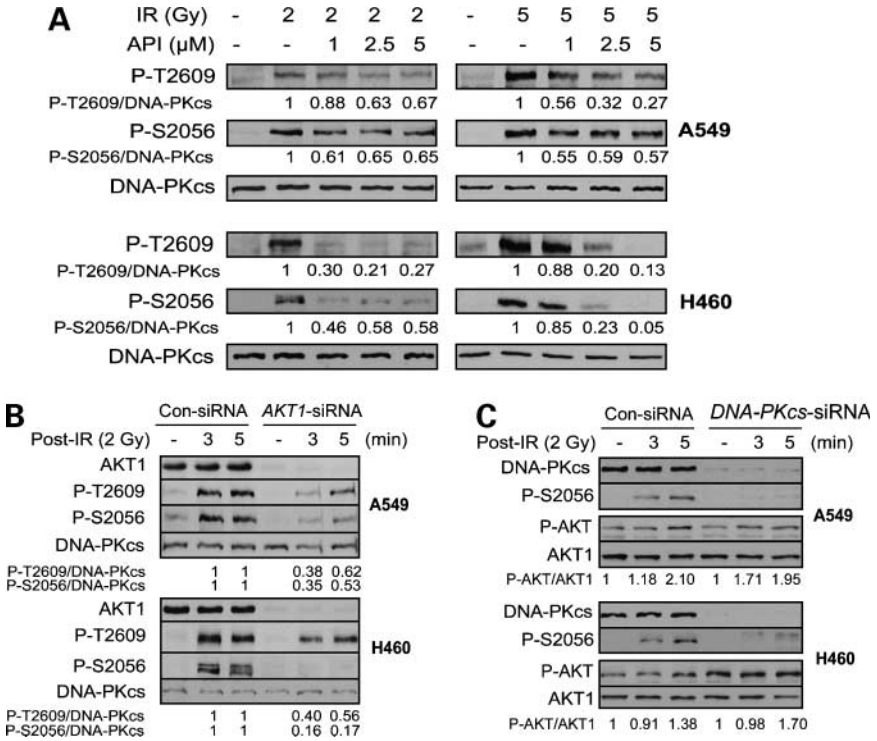

Figure 4. AKT targeting blocks IR-induced transphosphorylation/autophosphorylation of DNA-PKcs. **A,** A549 and H460 cells were treated with DMSO or API (1, 2.5, and 5 μmol/L) for 72 h and then exposed to single dose of IR (2 or 5 Gy). After irradiation for 5 min, cells were lysed and subjected to SDS-PAGE. Phosphorylation of DNA-PKcs at T2609 and S2056 was detected by using phosphospecific antibodies. Subsequently, blots were stripped and reprobed with total DNA-PKcs antibody. Densitometry values represent the ratio of P-DNA-PKcs/total DNA-PKcs (normalized to 1 in irradiated/API untreated condition). **B,** 4 d after siRNA transfection, cells were mock irradiated or irradiated with a single dose of 2 Gy. After cell lysis and electrophoresis, blots were probed for AKT1, P-T2609, and P-S2056. For loading control, total DNA-PKcs was detected. Densitometry values represent the ratio of P-DNA-PKcs/total DNA-PKcs (normalized to 1 in irradiated/control siRNA-transfected cells) at similar postirradiation time point. **C,** 4 d after siRNA transfection, cells were irradiated with a single dose of 2 Gy. From protein samples, P-DNA-PKcs (S2056), total DNA-PKcs, P-AKT (Ser^{472}/Ser^{473}), and total AKT were detected. Densitometry values represent the ratio of P-AKT (Ser^{472}/Ser^{473})/AKT (normalized to 1 in unirradiated transfected cells).

analyzed. As shown in Fig. 3B, the proportion of sub-G_1 H460 cells induced by combined API and radiation exposure could be repressed to levels of the untreated controls.

AKT Targeting Blocks IR-Induced Transphosphorylation/Autophosphorylation of DNA-PKcs in Lung Carcinoma Cell Lines A549 and H460

To address the question whether AKT1 targeting either by API or by specific AKT1-siRNA affects the activation of DNA-PKcs, we analyzed the transphosphorylation/autophosphorylation of this enzyme at the T2609 and S2056, respectively. Both sites are equally important for DNA-PKcs repair activity (31). As shown in Fig. 4A, the AKT1 inhibitor API markedly reduced T2609 and S2056 phosphorylation in irradiated A549 as well as H460 cells. For both cell lines, these results were confirmed by targeting AKT1 with specific siRNA (Fig. 4B). Interestingly, detection of total DNA-PKcs protein indicated that the level of DNA-PKcs is not affected either by API treatment or by siRNA transfection. As previous studies reported for glioblastoma cell lines that phosphorylation of AKT1 in response to insulin treatment is mediated through cytoplasmic DNA-PKcs (32), we knocked down DNA-PK expression in A549 and H460 cells by transfection with DNA-PK-specific siRNA. However, radiation-induced phosphorylation of AKT1 at Ser^{472}/Ser^{473} was similar in both control siRNA-transfected and DNA-PK-siRNA-transfected cells (Fig. 4C).

AKT1 Is Physically Associated with DNA-PKcs

Based on the results presented thus far, a direct interaction between AKT and DNA-PKcs by complex formation can be hypothesized. To investigate this, both cell lines were irradiated and immunoprecipitation of P-AKT was done with an antibody directed against the Ser^{472}/Ser^{473} epitope of AKT. As shown in Fig. 5A (*left*), coimmunoprecipitated DNA-PKcs phosphorylated at T2609 is detectable in both cell lines, indicating a direct physical interaction between AKT and DNA-PKcs. In an alternative experiment, coimmunoprecipitation of DNA-PKcs to immunoprecipitated AKT1 was shown (Fig. 5A, *right*). The radiation specificity of this complex formation was tested in a control experiment in which AKT phosphorylation at Ser^{472}/Ser^{473} was stimulated by EGF treatment. As indicated by the coimmunoprecipitation approach shown in Fig. 5A, regardless of EGF-induced phosphorylation of AKT at Ser^{472}/Ser^{473}, DNA-PKcs phosphorylated at T2609 could not be detected. Although complex formation was only detectable already under nonirradiated conditions, DNA-PKcs phosphorylation at T2609 can only be detectable after radiation exposure. Finally, as expected from the previous results, EGF, transforming growth factor-α, or amphiregulin as potent EGFR ligands did not induce T2609 phosphorylation of DNA-PKcs in unirradiated A549 and H460 cells (Fig. 5B).

Targeting of AKT Inhibits Repair of Radiation-Induced DNA-dsb

Because DNA-PKcs is the key enzyme of DNA-dsb repair via NHEJ, we further investigated whether API- or *AKT1*-siRNA-mediated inhibition of radiation-induced DNA-PKcs phosphorylation does impair DNA-dsb repair. Determining the number of residual γ-H2AX-foci 24 h after radiation exposure indicated a significant and radiation

dose-dependent increase in the amount of residual DNA-dsb either by API (Fig. 6A) or by AKT1-siRNA (Fig. 6B). Likewise, by analyzing γ-H2AX foci between 1 and 24 h after irradiation, a significant enhancement in residual DNA-dsb at time points 4, 8, and 24 h was observable (Fig. 6C). Thus, it could be postulated that interference with the direct interaction of AKT1 and DNA-PKcs clearly affects DNA-dsb repair. To test this further, the effect of AKT1-siRNA on residual γ-H2AX foci in cells cotransfected with DNA-PKcs-siRNA was analyzed after irradiation with 5 Gy. As shown in Fig. 6D, AKT1-siRNA efficiently knocked down AKT1 protein expression but did not affect DNA-PKcs expression. Likewise, transfection with DNA-PK-siRNA efficiently knocked down DNA-PKcs but did not affect the expression of AKT1, and as expected, cotransfection of both siRNA resulted in a nearly complete loss of the two proteins. The number of residual γ-H2AX foci after radiation exposure increased from ~10 per cell under control conditions to ~20 when cells were transfected with DNA-PK-siRNA. Transfection with AKT1-siRNA or cotransfection of AKT1-siRNA and DNA-PK-siRNA led to a nearly similar increase of γ-H2AX foci (Fig. 6D). The results of this experiment clearly support the idea that AKT targeting either by API or by siRNA does enhance residual DNA-dsb through inhibition of IR-induced DNA-PKcs activity.

Discussion

In the presented study, we show that a new selective inhibitor of the AKT pathway, API, does restore sensitivity to IR of the two radioresistant K-RAS mutated NSCLC cell lines. In both cell lines, targeting of the AKT pathway with API significantly enhanced the radiation toxicity. These results are consistent with the findings of Lee et al. (33), indicating that PI3K/AKT activity cooperates to stimulate survival of NSCLC cells. It is generally believed that inhibition of PI3K/AKT signaling sensitizes cells to radiotherapy through stimulating radiation-induced apoptosis (34, 35). However, at least for solid tumor cells, data

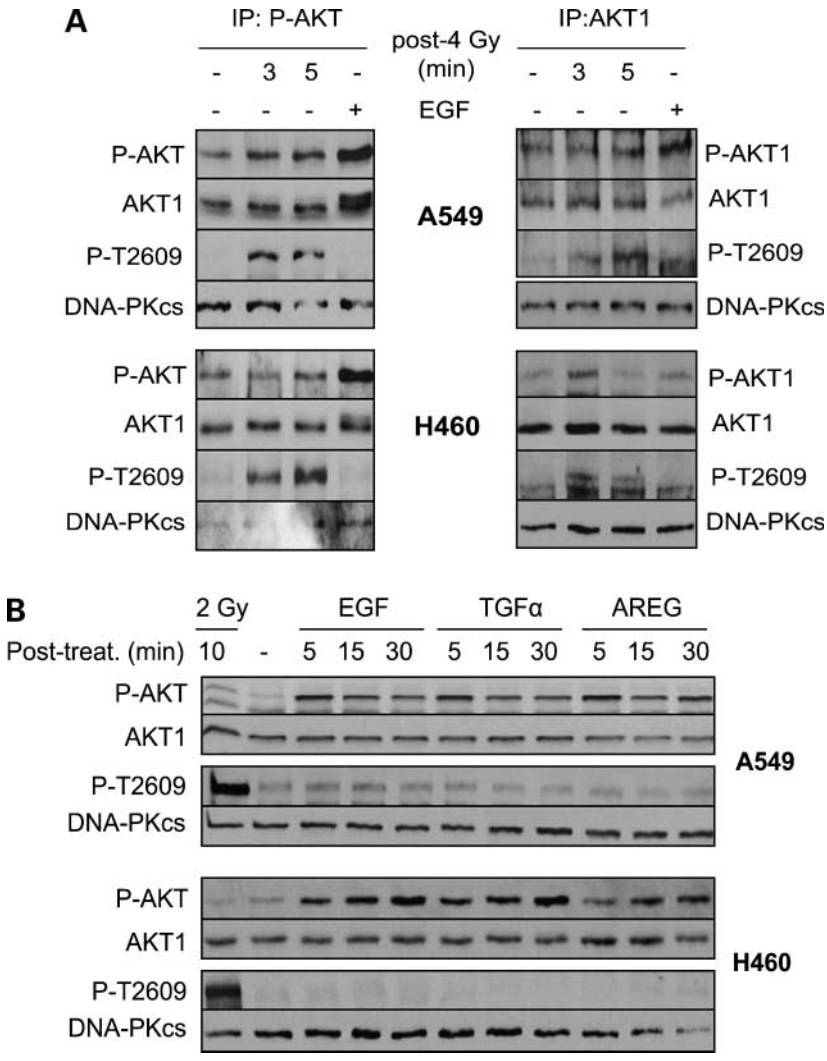

Figure 5. AKT1 is physically associated with DNA-PKcs. **A,** 48 h serum-starved cells were mock irradiated or irradiated with a single dose of 4 Gy and lysed at the time points indicated. Treatment with EGF (100 ng/mL, 5 min) was used as positive control. Immunoprecipitation of P-AKT (*right*) and AKT1 (*left*) was done as described previously (14). Coimmunoprecipitation of DNA-PKcs to either P-AKT or AKT1 was studied using P-DNA-PKcs (T2609) and DNA-PKcs antibodies. Subsequently, blots were stripped and reprobed with total AKT1 and DNA-PKcs antibodies. **B,** 48 h serum-starved cells were irradiated with a single dose of 2 Gy or treated with EGFR ligands EGF, transforming growth factor-α, and amphiregulin (100 ng/mL) for 5 min. From protein samples, phosphorylation of AKT (Ser^{472}/Ser^{473}) and DNA-PKcs (T2609) was detected. Subsequently blots were stripped and reprobed with total AKT1 and DNA-PKcs antibodies.

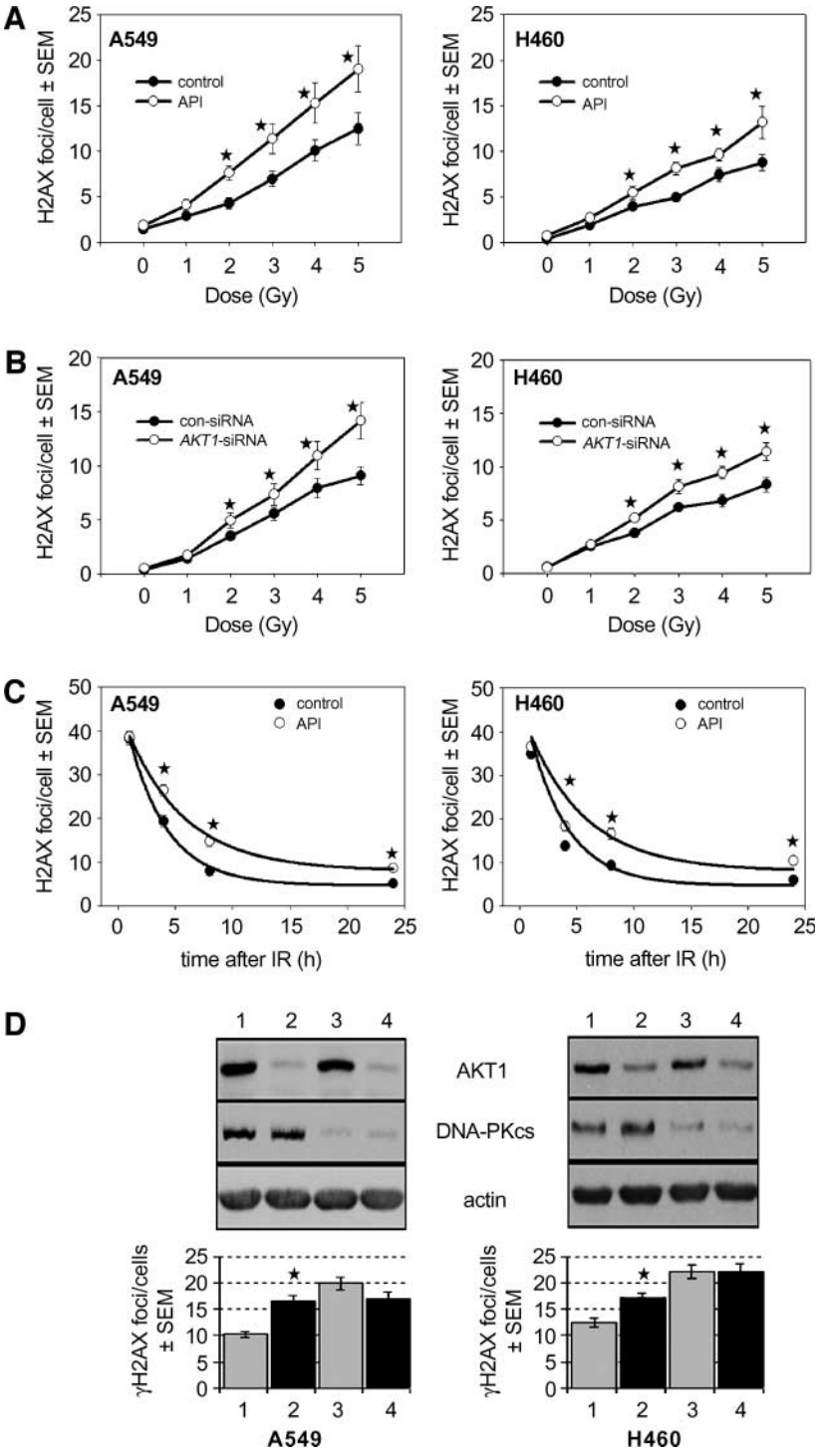

Figure 6. AKT1 targeting by API or *AKT1*-siRNA inhibits repair of radiation-induced DNA-dsb through blockage of DNA-PKcs activity. Cells grown on glass slides were pretreated with API (1 μmol/L; **A**) or transfected with *AKT1*-siRNA (**B**). Control cells were treated with DMSO or transfected with control-siRNA. After inhibitor treatment for 72 h and 4 d after siRNA transfection, cells were mock irradiated or irradiated with a single dose of 1 to 5 Gy. After irradiation for 24 h, cells were fixed and stained for γ-H2AX foci or nuclei (4′,6-diamidino-2-phenylindole). The number of γ-H2AX foci was counted for each treatment condition. *, $P < 0.05$, statistically significant difference between control and API- or *AKT1*-siRNA-treated cells (Student's *t* test). **C,** cells grown on glass slides were pretreated with API (1 μmol/L) for 72 h, irradiated with 2 Gy, and fixed at indicated hours after IR. Following staining for γ-H2AX foci or nuclei (4′,6-diamidino-2-phenylindole), the number of γ-H2AX foci was counted for each cell and the average number of foci/cell was recorded. *, $P < 0.05$, statistically significant difference between frequency of γ-H2AX in control compared with API-treated cells (Student's *t* test). **D,** cells were transfected with 50 nmol/L of following siRNA: *1,* control siRNA/control siRNA; *2,* control siRNA/*AKT*-siRNA; *3,* control siRNA/*DNA-PKcs*-siRNA; *4, AKT*-siRNA/*DNA-PKcs*-siRNA. After transfection for 4 d, cells were irradiated with 5 Gy. After irradiation for 5 min, cells were lysed and the level of DNA-PKcs and AKT1 was detected. Actin was detected as loading control. In a parallel experiment, 4 d after transfection with indicated siRNA, cells were irradiated. After irradiation for 24 h, frequency of residual γ-H2AX foci was analyzed. *, $P < 0.05$, statistically significant enhancement in residual γ-H2AX foci induced by *AKT*-siRNA (Student's *t* test).

exist that radiation-induced apoptosis is only marginally involved in radiation toxicity (36). Instead, for these cells, the reproductive cell death as a consequence of nonrepaired or misrepaired DNA damage is the major cause of radiation-induced cell death (37). Thus, by applying two cell lines with pronounced differences in their apoptotic behavior, we investigated whether the radiosensitizing effect of API is linked to radiation-induced apoptosis. As shown herein, radiation-dependent apoptosis was only induced to a significant degree in H460 but not in A549. Although thus far no data exist or are published that API may also have off-target effects, our results indicate the

specificity of API treatment to induce apoptosis only in apoptosis-sensitive H460 but not in apoptosis-resistant A549 cells. However, neither in A549 nor in H460 cells combined treatment with API and IR stimulated radiation-induced apoptosis to a significant degree. This is in agreement with earlier observations that NSCLC cells like many other cell lines isolated from solid tumors are relatively resistant to radiation-induced apoptosis (38–41). Thus, apoptosis induction in the lung carcinoma cell lines used in present study does not explain the radiosensitization effect of API. This is also reflected by the very similar dose-response curves of API-treated and untreated A549 and H460 cells (see Fig. 1), although API treatment resulted in a 6-fold increase in apoptotic cells in the cell line H460 but not in A549 (see Fig. 2). Thus, our findings clearly support the notion that the differential ability of cells from solid tumors to undergo apoptosis does not appear to play a significant role in determining clonogenic cell survival after exposure to IR (42, 43).

Survival of irradiated cells independent of their normal or malignant origin is largely dependent on the efficacy of repair of radiation-induced DNA-dsb (23, 37, 44). NHEJ is the dominant process in DNA-dsb repair and DNA-PKcs is the major enzyme required for this mechanism (45). Activation of DNA-PKcs is mediated through autophosphorylation or transphosphorylation at specific threonine and serine residues (T2609 and S2056; refs. 26, 46), and the ataxia telangiectasia mutated protein has been described to be responsible for transphosphorylation of the T2609 cluster (47, 48). The results presented herein provide the first direct evidence that AKT1 signaling in addition to ataxia telangiectasia mutated is essential for radiation-induced DNA-PKcs activity as a consequence of autophosphorylation and transphosphorylation at the S2056 and T2609 sites. Targeting of AKT1 either by the API inhibitor or by siRNA approaches strongly affected both S2056 and T2609 phosphorylation. Moreover, AKT targeting resulted in a significant impairment of DNA-dsb repair as indicated by the enhancement of residual γ-H2AX foci. This observation provides substantial evidence that blockage of DNA-dsb repair by AKT targeting is mediated through an inhibitory effect on DNA-PKcs. Furthermore, the results of the coimmunoprecipitation experiments using P-AKT-Ser^{472}/Ser^{473}, AKT1, and DNA-PKcs specific antibodies clearly showed that these proteins form a complex indicating a direct physical interaction between AKT1 and DNA-PKcs. Finally, the results of AKT1 targeting approaches suggested that, in cells exposed to IR, AKT1 may function in concert with ataxia telangiectasia mutated as a signaling kinase relevant for DNA-PK phosphorylation. Further experiments are necessary to clearly dissect the specific molecular mechanism of this protein-protein interaction for DNA-PKcs activation in cells exposed to IR.

Previous reports described a role for DNA-PK in the activation of AKT in response to different exogenous stimuli (by tumor necrosis factor-α, insulin, and CpG oligodeoxynucleotides; refs. 32, 49, 50). In the context of insulin-stimulated insulin-like growth factor-I receptor signaling, Feng et al. (32) have shown for human kidney cells that cytoplasmic DNA-PKcs can act as an upstream kinase responsible for the phosphorylation of AKT at Ser^{473}. Lu et al. (49) described that protein kinase C can stimulate AKT via DNA-PK activity to protect breast cancer cells against tumor necrosis factor-α-induced cell death. Finally, Dragoi et al. (50) reported that the activation of the AKT-dependent cell survival pathway by CpG oligodeoxynucleotides in immunocompetent cells is mediated by DNA-PK. These reports indicate an upstream function of DNA-PK in the activation of AKT. Our present findings that AKT is required to activate DNA-PKcs seem to be in conflict with these reports. However, these data were obtained under a completely different cellular scenario (that is, exposure to IR), which is known to activate EGFR/PI3K/AKT-mediated cell survival. Until now, no mechanistic evidence existed that radiation-induced EGFR signaling through PI3K/AKT is an essential step in stimulating DNA-dsb repair through activated DNA-PKcs. Thus, based on previous reports from Kao et al. (51) and our laboratory (15), the present study clearly indicates an AKT dependency of DNA-dsb repair in irradiated cells. As shown herein, stimulation of PI3K/AKT signaling to regulate DNA repair seems to be exclusively dependent on IR-induced DNA damage as the exogenous stimulus. This assumption is substantiated by the results showing that treatment of cells with EGFR ligands (EGF, transforming growth factor-α, and amphiregulin) did not result in stimulated DNA-PKcs phosphorylation. Likewise, radiation-induced phosphorylation of AKT was not impaired in cells transfected with DNA-PKcs-siRNA, indicating that DNA-PKcs is not needed for activating AKT in cells exposed to IR.

Taken together, in this study, we showed a complex formation of AKT and DNA-PKcs as an important step in the activation of DNA-PK-dependent DNA-dsb repair. Thus, targeting of AKT primarily sensitizes tumor cells through impairment of DNA-dsb repair and not through stimulation of radiation-induced apoptosis. These principal findings favor AKT as an excellent target to enhance radiation toxicity in tumor cells and may thus help to develop new effective strategies to overcome resistance of NSCLC to radiation therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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