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Role of the Phosphatidylinositol 3′-Kinase/PTEN/Akt Kinase Pathway in Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in Non-Small Cell Lung Cancer Cells

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ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)/APO-2L is a member of the TNF superfamily and has been shown to have selective antitumor activity. We here show that TRAIL does not induce apoptosis in some non-small cell lung cancer (NSCLC) cells. These cells are resistant to TRAIL because of the phosphatidylinositol 3′-kinase (PI3-K)-dependent activation of Akt/protein kinase B. The expression of phospho-Akt varies at the functional level but not at the mRNA level in NSCLC cells. Akt induces cell survival in NSCLC cells by blocking the Bid cleavage, upstream of cytochrome *c* release in the mitochondrial-dependent apoptotic pathway. The use of PI3-K inhibitors, Wortmannin or LY-294002, down-regulates the active Akt and reverses cellular resistance to TRAIL. In addition, genetically altering Akt expression by transfecting dominant negative Akt, sensitizes NSCLC cells to TRAIL. Conversely, transfection of constitutively active Akt into cells that express low, constitutively active Akt, increases TRAIL resistance. Alternate to this approach, transfection with PTEN, a lipid phosphatase, promotes sensitivity to TRAIL, whereas a PTEN mutant (PTEN-G129E) at the catalytic site is inactive in dephosphorylating active Akt. Furthermore, the loss of PTEN activity or overexpression of PI3-K-dependent Akt/protein kinase B activity promotes the survival of NSCLC cells. Modulation of Akt activity by combining pharmacological drugs or genetic alterations of the Akt expression induces cellular responsiveness to TRAIL. Thus, TRAIL can be used to treat NSCLC-resistant cells when combined with agents that down-regulate Akt activity.

INTRODUCTION

Lung cancer is the most frequent cause of cancer-related death in men and women in the United States and accounts for approximately more than a million deaths yearly worldwide (1, 2). NSCLCs[3] constitute 75% of primary lung cancers and are comprised of large-cell undifferentiated carcinomas, epidermoid carcinomas, and adenocarcinomas including bronchoalveolar lung cancers (3). Nearly 65% of NSCLCs exhibit significant heterogeneity, with 45% containing both adeno and squamous features (3, 4). NSCLCs arise via multistep mechanisms directly attributable to tobacco abuse. The explosion of knowledge regarding mechanisms of multistep pulmonary carcinogenesis, together with the availability of a variety of pharmaceutical agents that specifically target molecular defects in cancer cells, provide new opportunities for intervention in lung cancer patients (5, 6). However, the exact molecular mechanisms underlying the onset and progression of NSCLCs and the potential agents for therapy are still under active study. Recently, TRAIL/Apo-2L has been reported to be a potential candidate for cancer therapy (7).

Several groups including ours have shown that TRAIL induces apoptosis in many cancers and transformed cells (7–9). However, its proapoptotic effects are minimal in normal cells (9, 10; see comments in Ref. 9). TRAIL induces apoptosis by binding to its receptors DR4 and DR5, recruiting Fas-associated death domain, and forming death-inducing signaling complex (DISC; Ref. 11). This leads to the cleavage and activation of caspase-8 (12). Activation of caspase-8 by TRAIL leads to two different apoptotic pathways, depending on the cell type (7). In type I cells, TRAIL induces apoptosis in a mitochondrial-independent manner, activating downstream effector caspases such as caspase-3 and caspase-7 (13), whereas in type II cells, apoptosis proceeds via release of cytochrome *c* and Apaf-1 (7, 14). This results in activation of caspase-9, which then induces the execution phase of apoptosis. In addition, other mitochondrial proteins such as apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase (Smac/DIABLO) can induce apoptosis (15–17). Smac/DIABLO functions to promote caspase activation by inhibiting IAP (inhibitor of apoptosis) family proteins (16, 17).

It has been found that not all cancer cells are sensitive to TRAIL-induced apoptosis (18–20). Recently, based on *in vitro* experiments, TRAIL-resistant cancer cell lines have been discovered, although initially it was thought that resistance was mediated by expression of decoy receptors (7); intracellular components appear to be important in regulating the death and survival function, acting downstream of TRAIL receptors (12). Study of the intracellular mechanisms that control TRAIL resistance may enrich our knowledge of death receptor-mediated signaling and help to develop TRAIL-based approaches for cancer treatment. In fact, there are multiple survival factors operating intracellularly in response to survival signals. These signals may contribute to the development or progression of NSCLC, and understanding how these signals are important for survival and therapeutic resistance will render logical identification of drugs that abrogate these signals and induce apoptosis. Among the cellular signaling pathways that promote cell survival, Akt/PKB is one of the important survival factors that contributes resistance to apoptotic signals (21, 22).

Akt/PKB is a Ser/Thr protein kinase implicated in mediating a variety of biological responses, which includes the inhibition of apoptosis and the stimulating of cellular growth. Akt/PKB is activated in response to activation by many different growth factors, including insulin-like growth factor-I, epidermal growth factor, basic fibroblast growth factor, insulin, interleukin 3, interleukin 6, and macrophage-colony stimulating factor (21, 23). The Akt family of proteins contains a central kinase domain with specificity for Ser or Thr residues in substrate proteins. In addition, the NH_2 terminus of Akt includes a pleckstrin homology (PH) domain, essential for lipid-protein or protein-protein interactions. The Akt COOH terminus includes a hydrophobic and proline-rich domain. The primary structure of Akt is conserved across evolution, with the exception of the COOH-terminal tail, which is found in some, but not in all, species and isoforms. There are three mammalian isoforms of this enzyme: Akt1, Akt2, and Akt3.

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FBS, fetal bovine serum, XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl-2*H*-tetrazolium-5-carboxanilide inner salt; PKB, protein kinase B; RT-PCR, reverse transcription-PCR; CS, Cowden syndrome; DAPI, 4′,6-diamidino-2-phenylindole; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolyl carbocyanine iodide; CA, constitutively active; DN, dominant negative; WT, wild type; $\Delta\psi_m$, mitochondrial membrane potential.

Activation of all three of the isoforms is similar in that phosphorylation of two sites, one in the activation domain and the other in the COOH-terminal hydrophobic motif, are necessary for full activity (21). There are also survival stimuli that activate Akt via mechanisms that do not require stimulation of PI3-K. Akt activation may also be achieved through PI3-K-independent means, either through kinases such as Ca^{2+}/calmodulin-dependent protein kinase kinase (CAM-KK; Ref. 24) or cAMP-dependent protein kinase (PKA; Ref. 25), or under conditions of cellular stress (26–28). Activation of Akt by CAM-KK or by PKA does not appear to require phosphorylation of Ser-473. The relative importance of PI3-K-independent and -dependent means of Akt activation *in vivo* is unclear. On activation, Akt induces antiapoptotic effects through phosphorylation of Bad (29, 30) or caspase-9 (31), which directly regulates the apoptotic machinery or substrates such as human telomerase reverse transcriptase subunit (32), forkhead transcription family members, or IκB kinases (21), which indirectly inhibit apoptosis.

Activation of Akt/PKB is also negatively regulated by the tumor suppressor gene, *PTEN*, also known as *MMAC1* or *TEP1*. PTEN is a lipid phosphatase that can dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP_3; Refs. 34–36). Through direct regulation of PIP_3 levels, PTEN negatively regulates the PI3-K signaling pathway, which transduces extracellular growth-regulatory signals to intracellular mediators of growth and cell survival (37). Inactivation of PTEN by a genetic mutation results in increased Akt activity in many types of tumors including those of the endometrium, prostate, lung, and head and neck (33, 36–40).

The present study is aimed at examining the intracellular mechanisms of differential sensitivity of lung cancer cells to TRAIL. We demonstrate that H1155 cells express high levels of CA-Akt, which leads to resistance of TRAIL-induced apoptosis. Down-regulation of CA-Akt by PI3-K inhibitors or transfection with kinase-dead Akt or PTEN induces apoptosis in TRAIL-resistant H1155 cells. We also demonstrate that transfecting CA-Akt into A549 cells that have low endogenous Akt, increases resistance to TRAIL. We found that Akt interferes with Bid cleavage and maintains mitochondrial homeostasis, thereby providing resistance in the mitochondrial-dependent apoptosis pathway. The expression of Akt varied at the posttranscriptional level (phosphorylation) in NSCLC cells. These data suggest that agents that block activation of Akt can be used in combination with TRAIL to kill resistant cells.

MATERIALS AND METHODS

Reagents. Antibody against Bid was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). JC-1 dye was from Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence (ECL) Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). Antibody against phospho-Akt (Ser-473) was from New England Biolab (Beverly, MA). Wortmannin and LY-294002 were from Calbiochem (San Diego, CA). LipofectAMINE reagent was from Invitrogen Life Technologies (Carlsbad, CA). Caspase-8 and -9 kits were from Clonetech (Palo Alto, CA). All of the other chemicals used were of analytical grade and came from Fisher Scientific (Suwanee, GA) or Sigma (St. Louis, MO).

Cells and Culture Conditions. NSCLC cells (H23, A549, H125, H1155, and Calu-1 cells) were from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 supplemented with D-glucose, HEPES buffer, 2 mM L-glutamine, 1% penicillin-streptomycin mixture, and 10% FBS. H1155 cells were maintained in suspension culture in DMEM, supplemented with 1% penicillin-streptomycin and 10% FBS. All of the cells were maintained at 37°C with 5% CO_2.

Transient Transfection. Cells were plated in 60-mm dishes in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture at a density of 1×10^6 cells/dish. The next day, transfection mixtures were prepared. Cells were transfected with expression constructs encoding WT PTEN (pSG5L-HA-PTENwt), mutant PTEN (pSG5L-HA-PTEN-G 129E and pSGL5-HA-PTEN-G 129R), WT-Akt (pUSE-WT-Akt), CA-Akt (pUSE-CA-Akt), DN-Akt (pUSE-DN-Akt), FLAG-tagged DN caspase-9 (pcDNA3-DN-caspase-9), or the corresponding empty vectors (pSG5L, pUSE, or pcDNA3) in the presence of an expression vector pCMV-LacZ (Invitrogen Life Technologies) expressing β-galactosidase. The expression construct encoding FLAG-tagged DN-caspase-9 was a gift from Dr. Vishva M. Dixit (Genentech, Inc., South San Francisco, CA). The expression vectors encoding WT PTEN and mutant PTEN were kindly provided by Dr. W. Sellers (Harvard Medical School, Boston, MA), and WT-Akt, CA-Akt, and DN-Akt were from Upstate Biotechnology (Lake Placid, NY). For each transfection, 2 μg of DNA was diluted in 50 μl of medium without serum. After the addition of 3 μl of LipofectAMINE into 50 μl of Opti-MEM, the transfection mixture was incubated for 10 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added, and cultures were incubated for 24 h in the incubator. The next day, culture medium was replaced with fresh RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture, and TRAIL was added for 48 h. At the end of incubation, cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer. Expression of PTEN forms was assessed through immunoblot analysis with antibodies against the hemagglutinin (HA) epitope encoded for by the expression construct.

XTT Assay. NSCLC cells (1×10^4 in 100 μl of culture medium/well) were seeded in 96-well flat-bottomed plates, treated with or without drugs, and incubated for various times at 37°C and 5% CO_2. Before the end of the experiment, 50 μl of XTT labeling mixture (final concentration, 125 μM XTT and 25 μM PMS) per well were added, and the plates were incubated for 4 h at 37°C and 5% CO_2. The spectrophotometric absorbance was measured at 450 nm with a reference wavelength at 690 nm.

RT-PCR. Total RNA was extracted from NSCLC cells using the TRIZOL reagent protocol (Life Technologies, Inc.). The quality and quantity of the RNA was determined by measuring the absorbance of the total RNA at 260 and 280 nm, and by 1% agarose electrophoresis under reducing conditions. The one-step RT-PCR was used with the Platinum Taq kit (Life Technologies, Inc.) according to the manufacturer's instructions. Primers for Akt isoform expression were described (41) and were synthesized commercially (Life Technologies, Inc.). The primers for Akt1, Akt2, and Akt3 were: 5′-GCTGGACGATAGCTTGGA-3′ (Akt1 sense), 5′-GATGACAGATAGCTGGTG-3′ (Akt1 antisense), 5′-GGCCCCTGATCAGACTCTA-3′ (Akt2 sense), 5′-TCCTCAGTCGTGGAGGAGT-3′ (Akt2 antisense), 5′GCAAGTGGACGAGAATAAGTCTC-3′ (Akt3 sense), and 5′-ACAATGGTGGGCTCATGACTTCC-3′ (Akt3 antisense). β-Actin primers were 5′-GTGGGGCGCCCCAGGCACCA-3′ (sense), and 5′-CTCCTTAAGTCACGCACGATTTC-3′ (antisense). RT-PCR reactions contained 1 μg of total RNA, 0.2 μM primer, 1 μl of RT-Taq mix, and 25 μl of Reaction Mix. The cycling conditions for PCR were as follows: cDNA synthesis and predenaturation (1 cycle at 50°C for 30 min and at 94°C for 2 min); PCR amplification (30 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s. and extension at 68°C for 45 s). The PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide. The Akt primers were designed to generate 383- (Akt1), 276- (Akt2), and 329- (Akt3) bp products.

Measurement of Mitochondrial Energization. Retention of JC-1 was used as a measure of mitochondrial energization. Cells (5×10^5 in 500 μl of complete RPMI 1640 medium) were treated with drugs and incubated for various time points. JC-1 (40 nM) was added during the last 30 min of treatment. Cells were washed twice with HBSS to remove unbound dye. The concentration of retained JC-1 dye was determined by a fluorescence spectrometer.

Western Blot Analysis. Equal amounts of supernatant protein from the subcellular fractionation were resolved on 10% SDS-PAGE gels and electrophoretically transferred to PVDF membrane. The transferred membrane was blocked with 5% nonfat dry milk in TBST buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20] and incubated with primary antibody in TBST containing 0.5% BSA overnight at 4°C. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibody followed by chemiluminescent detection of immunoreactive proteins.

Apoptotic Index. NSCLC cells (1×10^4 cells) were seeded onto cover slips in Petri dishes and treated with TRAIL for 48 h. The treated cells were washed with PBS twice and stained with DAPI (0.5 μg/ml in PBS) for 1 h.

DAPI-stained cells were visualized under a fluorescent microscope (Nikon, Japan). Morphologically distinct cells were counted as apoptotic cells.

RESULTS

Effects of TRAIL on Cell Viability of NSCLC Cells and the Mechanism of TRAIL Resistance. The cytotoxic effects of TRAIL on NSCLC cells were compared by the XTT assay (Fig. 1*A*). Cell viability assays demonstrated that A549 and H125 cells were very sensitive to TRAIL, whereas H1155 cells were resistant to TRAIL. Because there are several factors involved in the resistance of TRAIL-induced cell death, we first examined the basal level of phospho-Akt in all of the NSCLC cells by immunoblot analysis. As shown in Fig. 1*B*, A459 cells expressed low phospho-Akt, whereas H1155 cells showed high expression of phospho-Akt. Thus, an inverse correlation was seen between cell death and CA-Akt level. H23 and Calu-1 cells showed equivalent levels of phospho-Akt levels, whereas H125 cells showed phospho-Akt level higher than A549 but lower than H23. The variation in the expression of Akt protein levels in NSCLCs prompted us to study the expression of Akt mRNA in all of the cells. According to a RT-PCR analysis, all three isoforms of Akt (Akt1, Akt2, and Akt3) were equally expressed in Calu-1, H1155, H125, A549, and H23 cells (Fig. 1*C*).

Effects of PI3-K Inhibitors on Akt Activity and Apoptosis on Lung Cancer Cells. Because Akt is an important cell survival regulator (42, 43), we explored whether PI3-K inhibitors can block the activation of Akt and sensitize the cells to TRAIL. Treatment of H1155 cells with LY-294002 (1 μM) or Wortmannin (1 μM) for 8 h reversed the high constitutive activity of Akt (Fig. 2*A*). Because the levels of phospho-Akt in H1155 cells inversely correlated with cell death, we sought to examine whether down-regulation of CA-Akt rendered H1155 cells sensitive to TRAIL. H1155 and A549 cells were pretreated with LY-294002 (1 μM) or Wortmannin (1 μM) for 4 h, followed by treatment with TRAIL (100 ng/ml for A549 and 200 ng/ml for H1155) for 48 h, and apoptotic nuclei were scored by DAPI staining (Fig. 2*B*). As expected, TRAIL alone induced apoptosis in A549 cells, but H1155 cells were resistant to apoptosis. Pretreatment with Wortmannin or LY-294002 further enhanced the effect of TRAIL in A549 cells, which expressed low active Akt. Wortmannin, LY-294002, and TRAIL alone did not induce apoptosis in H1155 cells. Pretreatment of H1155 cells with Wortmannin or LY-294002 induced apoptosis in H1155 cells when combined with TRAIL. Thus, the combination of Wortmannin or LY-294002 with TRAIL reduced the apoptotic resistance in H1155 cells.

Akt Induces TRAIL Resistance at the Level of Bid Cleavage in H1155 Cells. Earlier, we demonstrated that TRAIL induced apoptosis in the Type I (mitochondrial-independent) as well as Type II (mitochondrial-dependent) cells (11, 44). Therefore, we next examined the effects of TRAIL on Bid cleavage in H1155 and A549 cells (Fig. 2*C*). The Bid cleavage was assessed as a reduction in whole BID protein because the antibody recognized only whole Bid molecule, but not the cleavage product. TRAIL treatment resulted in reduction in whole Bid (indicating Bid cleavage) in A549 cells with or without Wortmannin or LY-294002. The reduction in Bid was not seen in H1155 cells treated with TRAIL alone, but was observed when

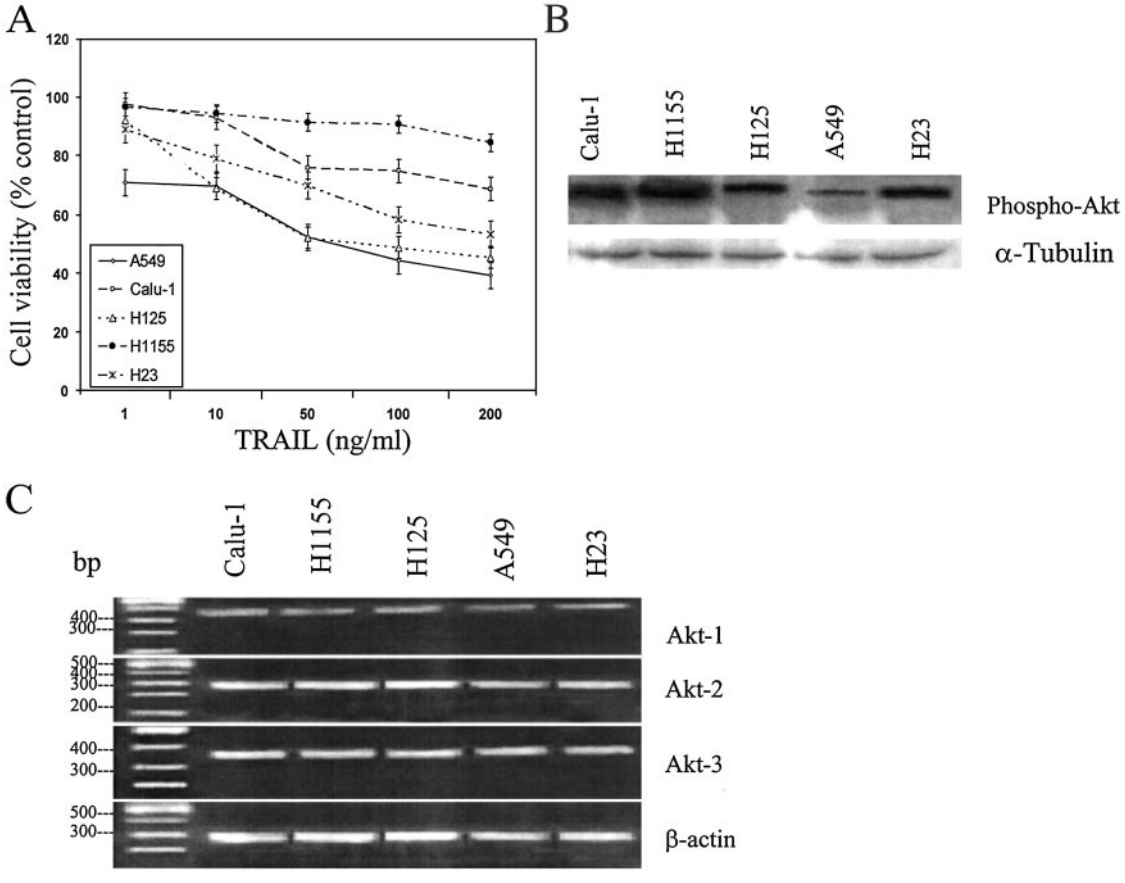

Fig. 1. Effects of TRAIL sensitivity on NSCLC cell lines and the mechanism of resistance. *A*, percentage of cell viability in Calu-1, H1155, H125, A549, and H23 cells treated with various doses of TRAIL for 48 h. Cell viability was determined by XTT assay. Data represent mean ± SE. *B*, immunoblot analysis of endogenous phospho-Akt in NSCLC cells. Cells were harvested and lysed, and the crude proteins were separated by 12% SDS-PAGE and probed with phospho-Akt antibody. α-tubulin was used as loading control. *C*, products of RT-PCR on 1% agarose gel electrophoresis for the expression of mRNA levels of Akt isoforms in NSCLC cells. β-actin was used as a loading control.

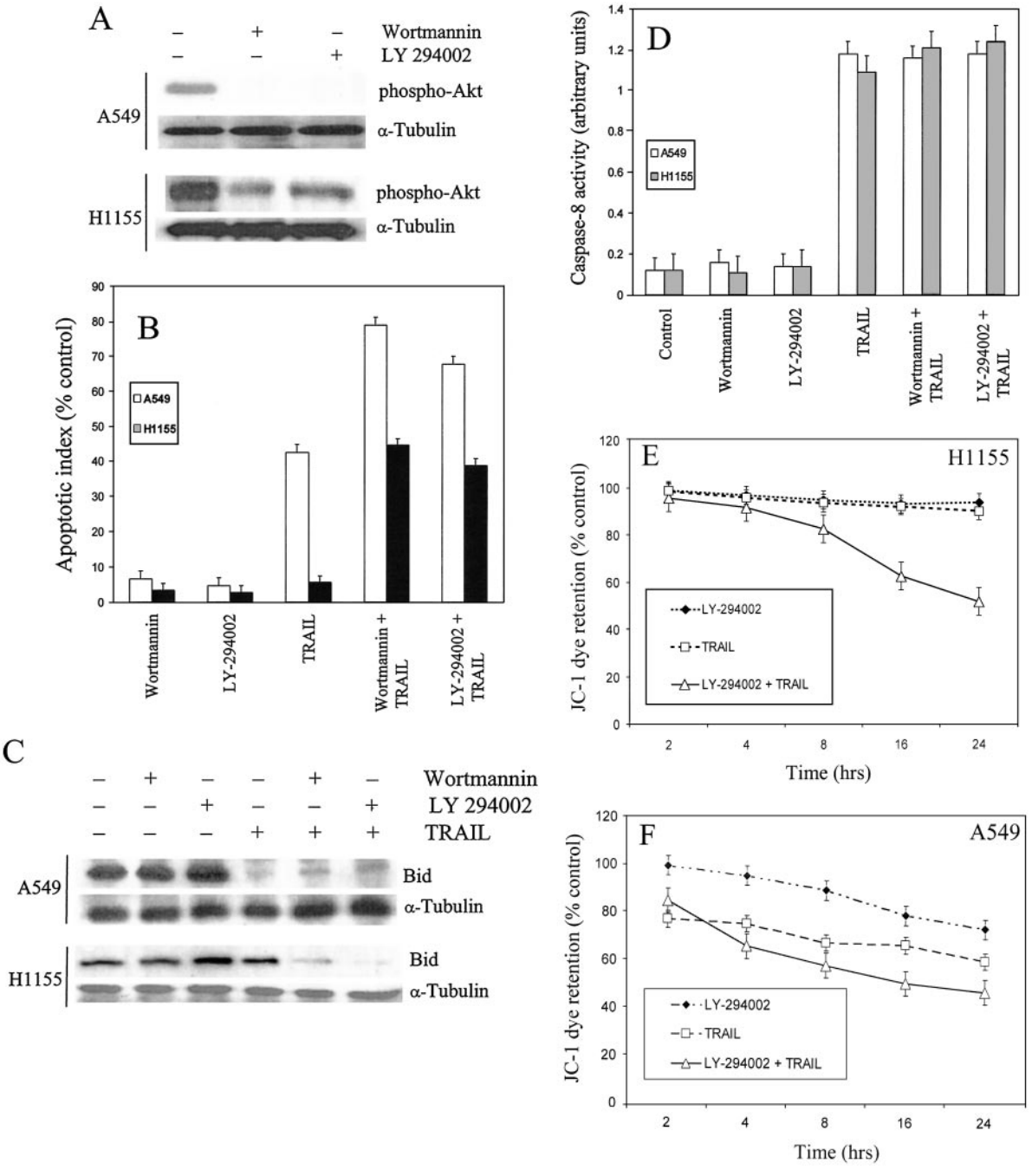

Fig. 2. Effect of Wortmannin, LY-294002, and TRAIL on apoptosis, Bid cleavage, caspase-8 activity, and $\Delta\psi_m$ in A549 and H1155 cells. *A*, immunoblot analysis of phospho-Akt levels in A549 and H1155 cells treated with Wortmannin (1 μM) or LY-294002 (1 μM) for 8 h. *B*, apoptotic index in A549 and H1155 cells, pretreated with Wortmannin (1 μM) or LY-294002 (1 μM) for 4 h followed by TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) for 48 h. *C*, immunoblot analysis for the assessment of Bid cleavage in A549 and H1155 cells, treated with Wortmannin (1 μM) or LY-294002 (1 μM) in the presence or absence of TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) for 18 h. Crude proteins of the harvested cells were separated by 12% SDS-PAGE, transferred to PVDF membrane and probed with Bid antibody. α-tubulin was used as loading control. *D*, caspase-8 activity in A549 and H1155 cells, treated with Wortmannin (1 μM) or LY-294002 (1 μM) in the presence or absence of TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) for 8 h. Caspase-8 activity was assessed as per the Clontech kit. *E* and *F*, $\Delta\psi_m$ in A549 and H1155 cells treated with LY-294002 (1 μM) in the presence or absence of TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) at various time points. Membrane potential was measured by JC-1 dye retention using fluorescence spectrophotometer and was shown in (*E*) H1155 cells and (*F*) A549 cells.

TRAIL was combined with Wortmannin or LY-294002. Because the reduction in Bid was not seen in H1155 cells treated with TRAIL alone, we explored the molecules upstream of Bid to rule out the possibility of defects in caspase-8 activity. There was no difference in TRAIL-induced caspase-8 activity between H1155 and A549 cells (Fig. 2*D*), which indicated that active Akt does not interfere with signals upstream of Bid. Preincubation of cells with Wortmannin or LY-294002 did not affect TRAIL-induced caspase-8 activity. These data suggest that resistance induced by CA-Akt interferes with Bid cleavage, but not in caspase-8 activation in H1155 cells.

Akt Attenuates TRAIL-induced Drop in $\Delta\psi_m$ in H1155 Cells. Mitochondria appear to play a central role in apoptosis and have been a major focus of recent studies (14, 45). During apoptotic cell death, the early events that occur are mitochondrial depolarization and loss of cytochrome *c* from the mitochondrial intermembrane space (14, 45). The fluorescent dye JC-1 localizes to the mitochondria, and the

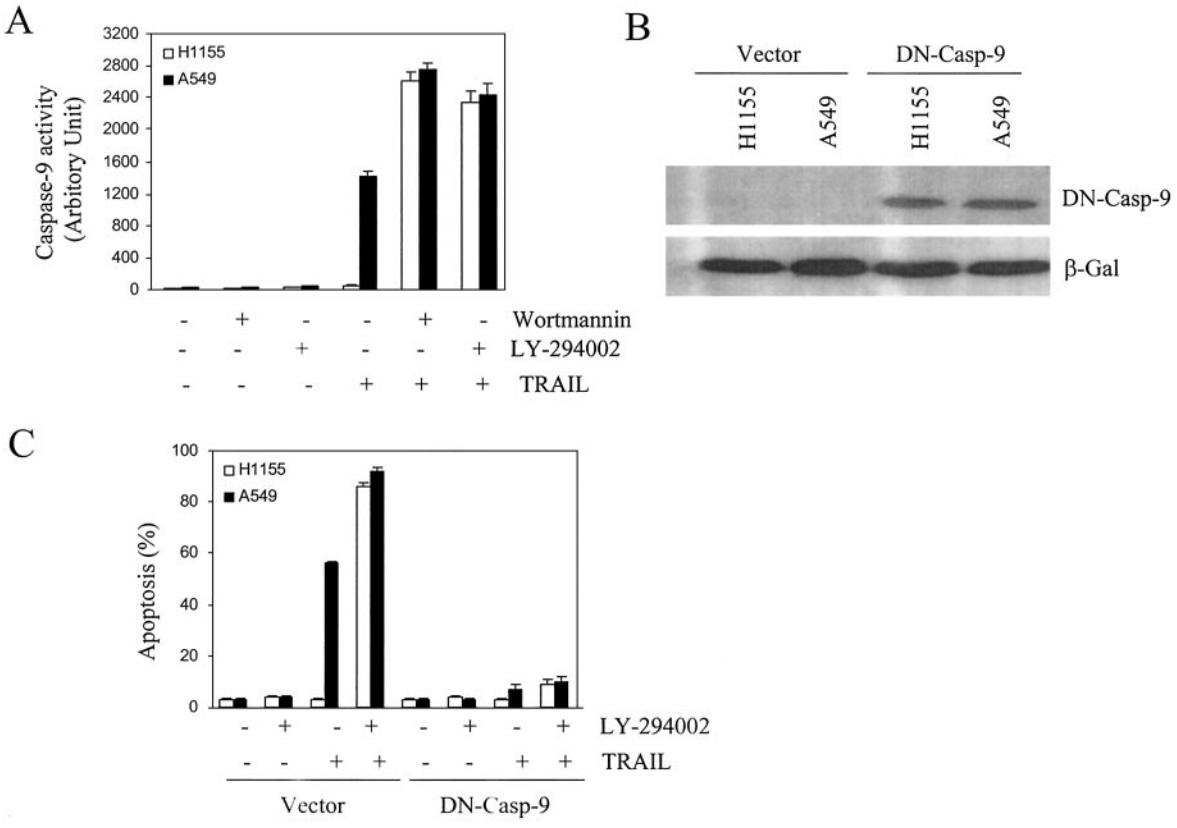

Fig. 3. Effect of TRAIL, Wortmannin, and/or LY-294002 on caspase-9 activity in A549 and H1155 cells. *A*, regulation of caspase-9 activity by Wortmannin plus TRAIL or LY-294002 plus TRAIL. H1115 and A549 cells were treated with Wortmannin (200 nM) or LY-294002 (20 μM) in the presence or absence of TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) for 18 h. Caspase-9 activity was measured as per manufacturer's directions (Oncogene Research Products). The data represent mean ± SE. *B*, overexpression of DN-caspase-9 (*DN-Casp-9*) in H1155 and A549 cells. Cells were transfected with either DN-caspase-9 cDNA or an empty vector along with a control plasmid (pCMV-LacZ) encoding β-galactosidase (β-Gal). The DN-caspase-9 protein was detected by the Western blot analysis with an anti-Flag antibody. *C*, effects of DN-caspase-9 on TRAIL-induced apoptosis in H1155 and A549 cells. Cells were transfected as described above in Fig. 3*B*. After transfection, cells were treated with LY-294002 (1 μM) in the presence or absence of TRAIL (100 ng/ml for A549 cells and 200 ng/ml for H1155 cells) for 48 h. Apoptosis was measured by DAPI staining.

mitochondrial permeability transition (MPT) reduces its accumulation as a consequence of loss in $\Delta\psi_m$. If CA-Akt causes TRAIL resistance in H1155 cells, then Akt may block apoptosis either upstream or downstream of mitochondrial dysfunction such as $\Delta\psi_m$ and cytochrome *c* release from the mitochondria. Because TRAIL-induced Bid cleavage in H1155 cells occurs in the presence of Wortmannin or LY-294002, we sought to investigate mitochondrial dysfunction by measuring $\Delta\psi_m$. Treatment of cells with TRAIL or LY-294002 had no effect on $\Delta\psi_m$ in H1155 cells (Fig. 2*E*). However, TRAIL in combination with LY-294002 caused a rapid decrease in $\Delta\psi_m$ in H1155 cells. In contrast, treatment of A549 cells with TRAIL alone or TRAIL plus LY-294002 caused a significant drop in $\Delta\psi_m$ (Fig. 2*F*).

Down-Regulation of Caspase-9 Inhibited TRAIL and/or LY-294002-Induced Apoptosis. Mitochondrial dysfunction appears to be essential for the formation of apoptosomes (a complex consisting of cytochrome *c*, Apaf-1, and ATP), which, in turn, activate caspase-9 and downstream effector caspases (14, 45). We, therefore, sought to examine the activation of caspase-9 in cells treated with TRAIL in the presence or absence of Wortmannin or LY-294002. Treatment of H1155 and A549 cells with Wortmannin or LY-294002 had no effect on caspase-9 activity (Fig. 3*A*). TRAIL induced caspase-9 activity in A549 cells but not in H1155 cells. Wortmannin or LY-294002 further enhanced TRAIL-induced caspase-9 activation in both cell types.

Because pretreatment of H1155 cells with Wortmannin or LY-294002 resulted in caspase-9 activation by TRAIL, we sought to examine the effects of a DN caspase-9 on apoptosis. H1155 and A549 cells were transfected with either a DN-caspase-9 cDNA or an empty vector along with a control plasmid (pCMV-LacZ) encoding the β-galactosidase. Fig. 3*B* showed the overexpression of DN-caspase-9 in H1155 and A549 cells. As noticed before, H1155 cells were resistant to TRAIL-induced apoptosis, whereas A549 cells were sensitive (Fig. 3*C*). Pretreatment of H1155 and A549 cells with LY-294002 resulted in TRAIL-induced apoptosis. Overexpression of DN-caspase-9 in A549 cells inhibited apoptosis induced by TRAIL and/or LY-294002. Similarly, overexpression of DN-caspase-9 in H1155 cells inhibited apoptosis induced by TRAIL plus LY-294002. These data suggest that events downstream of mitochondria are intact in H1115 cells, and resistance to TRAIL occurs at the level of Bid cleavage.

Attenuation of CA-Akt by DN-Akt Sensitizes H1155 Cells to TRAIL. Because our earlier experiments demonstrated that PI3-K inhibitors sensitize H1155 cells to TRAIL, we used a genetic approach to down-regulate CA-Akt. If Akt is the signaling molecule for TRAIL resistance in H1155 cells, then down-regulation of Akt by DN-Akt would sensitize these cells to TRAIL. We transiently transfected cells with empty vector or with WT-Akt, CA-Akt, or DN-Akt, and treated with or without TRAIL. Immunoblot analysis confirmed the transfection and showed low levels of phospho-Akt in cells transfected with DN-Akt (Fig. 4*A*). Transfection of H1155 cells with empty vector or with WT-Akt or CA-Akt had no effect on apoptosis, whereas transfection of cells with DN-Akt induced sensitivity to TRAIL (Fig. 4*B*). By comparison, transfection with empty vector, WT-Akt, or DN-Akt in A549 cells had no significant effect on TRAIL-induced apoptosis, whereas transfection with CA-Akt abrogated the TRAIL-induced ap-

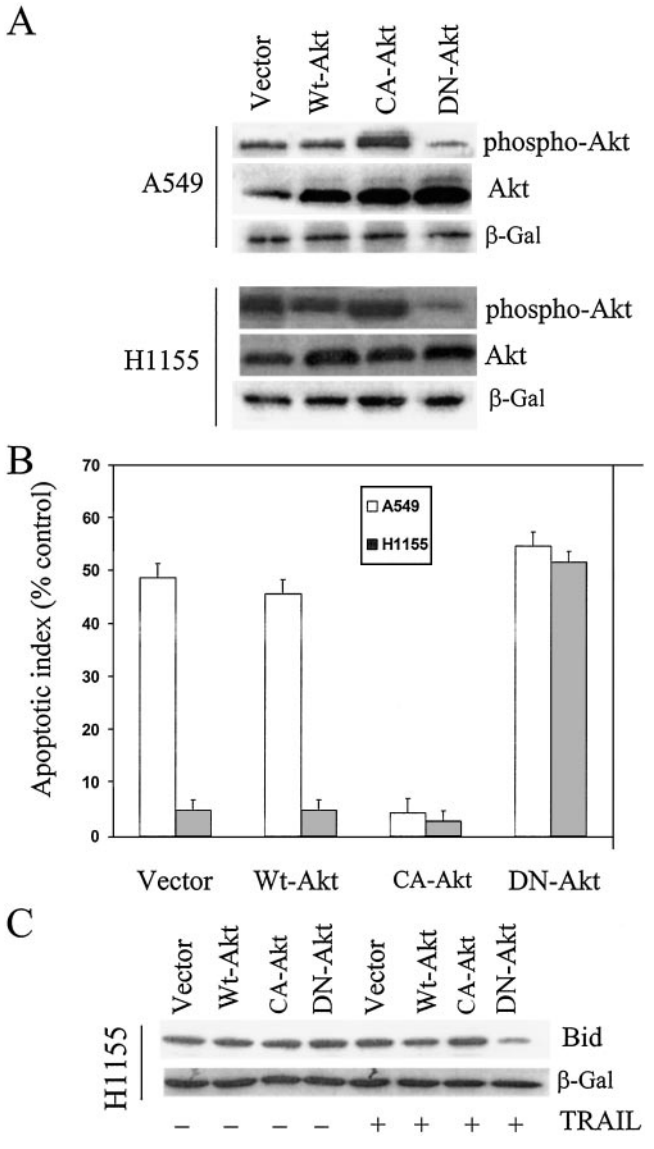

Fig. 4. Effect of CA-Akt and DN-Akt on TRAIL- induced apoptosis and Bid cleavage. *A*, immunoblot analysis of phospho-Akt levels in A549 cells and H1155 cells transiently transfected with empty vector, WT-Akt, CA-Akt, or DN-Akt. In addition, cells were cotransfected with control plasmid (pCMV-LacZ) encoding the β-galactosidase (*β-Gal*) enzyme. *B*, apoptotic index of A549 and H1155 cells, treated with or without TRAIL (100 ng/ml for A549 and 200 ng/ml for H1155 cells) for 48 h. Apoptosis was measured by DAPI staining of the nuclei. Data represent three individual experiments performed in triplicate. *C*, immunoblot analysis of Bid cleavage in H1155 cells transiently transfected with empty vector, WT-Akt, CA-Akt, or DN-Akt. In addition, cells were cotransfected with control plasmid (pCMV-LacZ) encoding the β-gal enzyme. Cells were treated with or without TRAIL (200 ng/ml) for 48 h. Cells were harvested and lysed, and the crude proteins were separated, and immunoblot analysis was performed for Bid and His-tagged β-gal as described under "Materials and Methods." More than 80% of the cells were transfected, and there were no differences in transfection efficiency among groups.

optosis (Fig. 4*B*). The data suggest that an increase in CA-Akt in A549 cells, but not in WT-Akt, alters TRAIL sensitivity. Furthermore, down-regulation of CA-Akt by DN-Akt in H1155 cells resulted in a reduction in the Bid level in response to TRAIL treatment (Fig. 4*C*), which suggests that attenuation of CA-Akt in H1155 cells is sufficient to induce apoptosis by TRAIL via Bid cleavage.

PTEN Sensitizes H1155 Cells to TRAIL. The tumor suppressor protein, PTEN, is a member of the mixed function, Ser/Thr/Tyr phosphatase subfamily of protein phosphatases. Its physiological substrates are primarily 3-phosphorylated inositol phospholipids, which are products of PI3-K (33, 35). These studies suggest that downstream targets of the PI3-K pathway, such as Akt, are negatively regulated by PTEN. *PTEN* gene is inactivated in many common malignancies, including glioblastoma and endometrial, lung, and prostate cancer (35, 40, 46). Mutations in PTEN are reported in a variety of human cancers (33, 36, 37, 40) and, hence, PTEN mutants can be used as an indicator to assess the lipid phosphatase activity. We have included PTEN mutants (PTEN-G129E and PTEN-G129R) in the study to assess the role of the catalytic motif of PTEN in rendering lipid phosphatase activity. We, therefore, transiently transfected H1155 cells with empty vector, PTEN-wt, and mutated PTEN (PTEN-G129E and PTEN-G129R) and incubated in the presence or absence of TRAIL. Transfection of H1155 cells with PTEN-wt resulted in an induction of apoptosis (Fig. 5, *A* and *B*) and whole Bid (Fig. 5*C*) on treatment with TRAIL. Transfection with mutant PTEN (G129E) rendered cells resistant to TRAIL and had no affect on whole Bid level, which indicates that mutation in PTEN abrogates its phosphatase activity. Transfection of H1155 cells with PTEN-G129R, which has less phosphatase activity compared with PTEN-wt, showed increased apoptosis and reduced whole Bid level (because of cleavage), which indicates that they are less resistant to TRAIL compared with PTEN-G129E. These data confirmed our previous findings that CA-Akt in prostate cancer is involved in the resistance of LNCap cells to TRAIL (44).

DISCUSSION

In the present study, we demonstrated that the Akt/PKB signaling pathway plays an essential role in regulating cells to escape from TRAIL-induced apoptosis. There are a variety of reports suggesting the role of Akt in chemotherapeutic resistance to apoptosis and indicating its prosurvival function (44, 47). However once expressed, active Akt is under tight regulation by PI3-K and other kinases of the signaling pathway that promote cell survival. Signaling of growth factors translocates Akt to the inner surface of the plasma membrane in proximity to regulatory kinases that phosphorylate and activate Akt (21). Indeed, Akt regulates a number of intracellular components implicated in cell growth and survival. We found that mRNA expression of Akt isoforms are the same in all NSCLC cells when indicated by RT-PCR that transcriptional regulation is not altered; however, the mRNA functional expression changes at the posttranslational level, consistent with reports by others (47, 48). This suggests that the overexpression of Akt protein is tissue-specific; and we also observed variations in the levels of Akt expression in the five NSCLC cells tested. Expression of Akt1 has been observed in a human gastric cancer (49), Akt2 in ovarian and pancreatic cancers (26, 50), and Akt3 in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines (51). The expression of Akt at the protein level depends on activation of upstream kinases such as PI3-K or PDK1, and/or low expression of lipid phosphatases such as PTEN that down-regulate active Akt (21).

Most of the signals for survival function trigger growth factor receptors, which activate the PI3-K/Akt pathway and promote cell growth (52). Because PI3-K targets Akt for survival, we have modulated the activation Akt by two approaches. In the first, we used the PI3-K inhibitors Wortmannin and LY-294002 to sensitize H1155 cells to TRAIL. Wortmannin and LY-294002 have been reported to inhibit Akt/PKB phosphorylation at Thr 308 and Ser-473 (47, 53). Although A549 cells undergo cell death on treatment with TRAIL alone, increased cell death of H1155 cells was observed only when combined with Wortmannin or LY-294002. This directly correlates with the apoptotic index, because we found increased apoptosis in TRAIL plus Wortmannin-treated H1155 cells and in TRAIL plus LY-294002-treated H1155 cells. This suggests that a high level of CA-Akt in H1155 cells is responsible for TRAIL resistance.

It is known from several reports that active Akt inhibits apoptosis

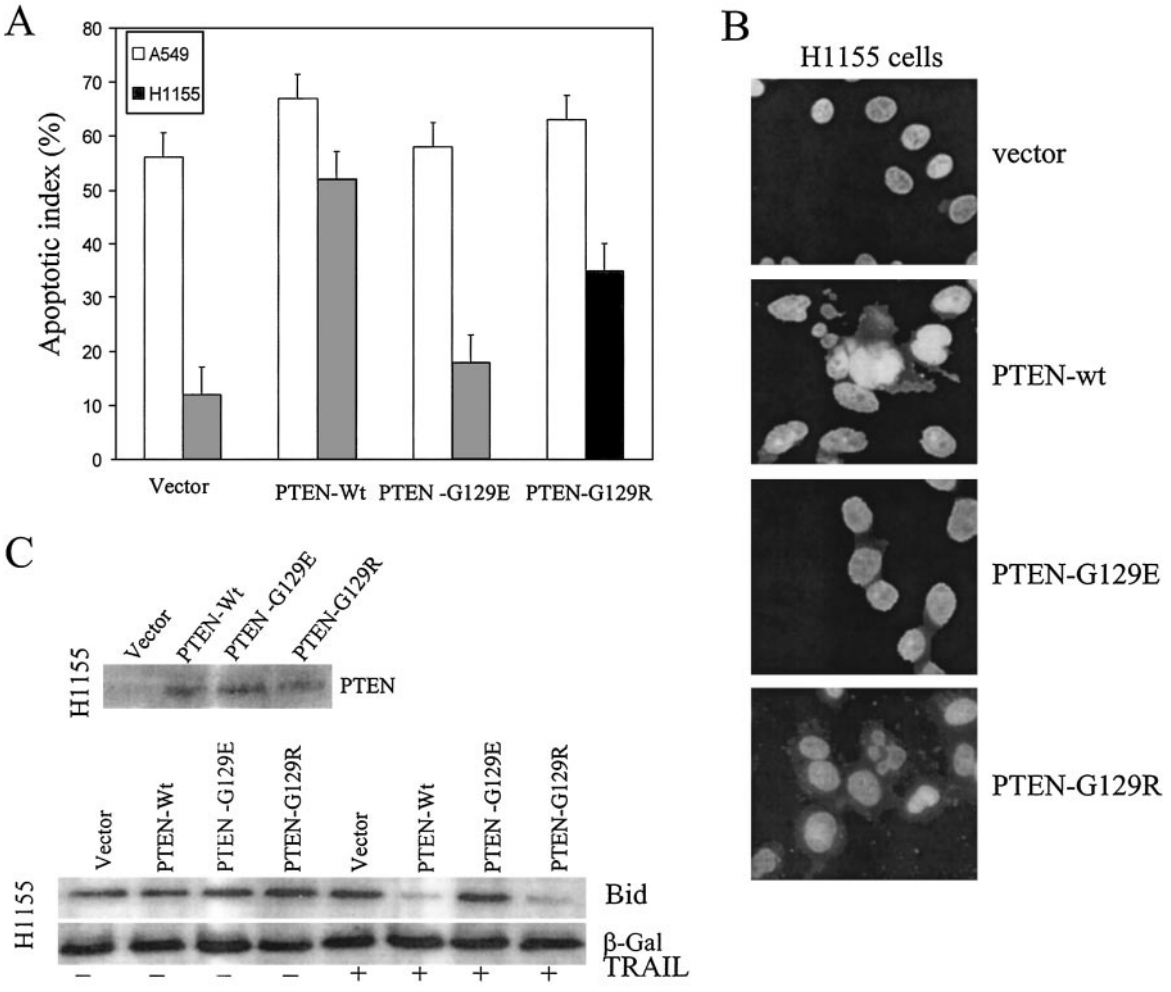

Fig. 5. Overexpression of PTEN sensitizes H1155 cells to TRAIL-induced apoptosis. *A*, apoptotic index of A549 and H1155 cells transiently transfected with empty vector, PTEN-Wt, PTEN-G129E, or PTEN-G129R mutant in the presence of control plasmid (pCMV-LacZ) encoding the β-galactosidase (*β-Gal*) enzyme. Cells were treated with or without TRAIL (100 ng/ml for A549 and 200 ng/ml for H1155 cells) for 48 h. Data represent three individual experiments performed in triplicate. *B*, apoptosis was measured by DAPI staining, and the morphology of TRAIL-treated H1155 cells. *C*, immunoblot analysis of H1155 cells transiently transfected with empty vector, PTEN-Wt, PTEN-G129E, or PTEN-G129R in the presence of control plasmid (pCMV-LacZ) encoding the β-galactosidase (*β-Gal*) enzyme. After transfection, cells were treated with or without TRAIL (200 ng/ml) for 48 h. Cells were harvested and lysed; the crude proteins were separated; immunoblot analysis was performed for Bid and His-tagged β-galactosidase (*β-Gal*) as described under "Materials and Methods." More than 80% of the cells were transfected, and there were no differences in transfection efficiency among groups.

by blocking Bid cleavage that is essential for releasing cytochrome *c* from the mitochondrial intermembrane space (21, 29). We found that H1155 cells did not show Bid cleavage on TRAIL treatment alone, whereas TRAIL plus Wortmannin or TRAIL plus LY-294002 induced Bid cleavage. Because truncated Bid (tBid) translocates to mitochondria to release cytochrome *c* (54), it is likely that Akt indirectly participates in the inhibition of cytochrome *c* release confirming earlier studies by ourselves (44) and others (55–57). Active Akt inhibits the drop in $\Delta\psi_m$ in H1155 cells. Whereas ineffective alone, TRAIL in combination with Wortmannin or LY-294002 induced a drop in $\Delta\psi_m$, opened the permeability transition pore to release cytochrome *c*, and subsequently activated caspase-9 in H115 cells. However, Akt cannot inhibit apoptosis induced by microinjection of cytochrome *c* (57). In fact, Akt inhibits apoptosis and cytochrome *c* release induced by several proapoptotic Bcl-2 family members (29, 57, 58). Taken together, Akt promotes cell survival by intervening in the apoptosis cascade upstream of cytochrome *c* release and downstream of caspase-8 activation via a mechanism involving mitochondria in apoptotic cells (type II cells).

In addition to the use of PI3-K inhibitors, we used a genetic approach to down-regulate active Akt by transfecting kinase-dead Akt (DN-Akt). Down-regulation of Akt by DN-Akt transfection rendered H1155 cells susceptible to TRAIL-induced apoptosis. On the other hand, up-regulation of Akt activity in A549 cells, which express very low CA-Akt, restored TRAIL resistance. Thus, genetic manipulations or rendering active-Akt to inactive-Akt in NSCLC cells sensitizes them to TRAIL, which confirms previous findings with prostate cancer (44).

Additional support for the involvement of Akt in NSCLC comes from the functional contribution of PTEN. The *PTEN/MMAC* tumor suppressor gene, is a lipid phosphatase that dephosphorylates PI3-K-generated 3′-phosphorylated phosphatidylinositides *in vivo* (35, 36). It has been shown that $PTEN^{-/-}$ mice have elevated levels of 3′-phosphorylated phospholipids and die during embryogenesis as a result of a failure in developmental apoptosis (40, 59). A recent study reported that A549 cells express high PTEN activity, whereas it is absent in H1155 cells because of nonsense mutations (47). Because PTEN down-regulates Akt, we intended to study PTEN function by transfecting H1155 cells that express high levels of active Akt. The transfected PTEN attenuated Akt function and rendered H1155 cells sensitive to TRAIL, which suggested that Akt acts downstream of PTEN and can be dephosphorylated by reintroducing PTEN-wt. Our study also demonstrated that the introduction of mutant PTEN rendered insensitive to TRAIL because we observe a reduced apoptotic

index in PTEN-G129E-transfected H1155 cells. Mutations in PTEN are found in several human tumors and in hamartomatous syndromes, which includes CS (60, 61). Furthermore, CS has a PTEN-G129E mutation that changes a Gly residue in the catalytic signature motif to a glutamate, abolishing the tumor-suppressor activity of PTEN (62). Therefore, this mutation can be used as an important indicator to determine whether a proposed function of PTEN is specific for its role as a tumor suppressor. Our transfection study with PTEN-G129E in A549 and H1155 cells demonstrated that the PTEN catalytic site is essential in rendering the lipid phosphatase activity, because we observed a decline in apoptotic index in H1155, whereas A549 cells show nonsignificant reduction in mutant-transfected cells compared with transfection with PTEN-wt. Although there are reports stating that PTEN-G129E and PTEN-G129R failed to induce a G_1 block in renal carcinoma cells (63), surprisingly, we found that transfection with PTEN-G129R in H1155 cells showed higher apoptotic cells compared with PTEN-G129E transfection. This is supported by the study that transfection with PTEN-G129E did not show Bid cleavage, but PTEN-wt and PTEN-G129R showed cleavage of Bid, which suggests that Akt interferes at the level of Bid cleavage and mutant PTEN-G129E failed to inhibit the Akt phosphorylation in H1155 cells. Although PTEN-G129E mutants are seen in CS, it is less clear in PTEN-G129R mutation, because we observe less resistance to TRAIL-induced apoptosis. It may be possible that the mutation that changes Gly to Arg in the catalytic site is ineffective in complete abrogation of PTEN activity. However, tumor cells that lack PTEN activity might be predicted to harbor excessive Akt activity. These studies suggest that the PI3-K/Akt pathway is involved more in oncogenic transformation, cell cycle progression, and cellular resistance to apoptosis.

It is suggested that the cytotoxic effects of TRAIL on lung cancer cells vary significantly and inversely correlate with the levels of CA-Akt. Our study regarding the clinical implications of using TRAIL in lung cancer therapy depends on cells expressing CA-Akt, because they are more resistant to undergoing apoptosis by TRAIL. Down-regulation of CA-Akt by pharmacological or genetic approaches altered the cellular responsiveness to TRAIL. Thus, TRAIL in combination with agents that down-regulate Akt activity can have clinical applicability in treating lung cancer cells that are resistant to chemotherapy.

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