[CANCER RESEARCH 63, 6697–6706, October 15, 2003]

Adenoviral-Mediated Expression of a Kinase-Dead Mutant of Akt Induces Apoptosis Selectively in Tumor Cells and Suppresses Tumor Growth in Mice

Amanda Jetzt, John A. Howe, Mark T. Horn, Eugene Maxwell, Zhizhang Yin, Duane Johnson, and C. Chandra Kumar[1]

Department of Tumor Biology, Schering-Plough Research Institute, Kenilworth, New Jersey 07033 [A. J., E. M., Z. H., C. C. K.], and Canji, Inc., La Jolla, California 92121 [J. A. H., M. T. H., D. J.]

ABSTRACT

Akt/protein kinase B is a serine/threonine kinase that plays a critical role in cell survival signaling, and its activation has been linked to tumorigenesis in several human cancers. Up-regulation of Akt, as well as its upstream regulator phosphatidylinositol 3-kinase, has been found in many tumors, and the negative regulator of this pathway, mutated in multiple advanced cancers suppressor (MMAC; also known as phosphatase and tensin homologue deleted on chromosome 10), is a tumor suppressor gene. We have investigated the effects of inhibiting Akt signaling in tumor cells by expression of an Akt kinase-dead mutant in which the two regulatory phosphorylation sites have been mutated to alanines. This mutant, which functions in a dominant negative manner (Akt-DN), was introduced into tumor cells using a replication-defective adenovirus expression system. As controls we used adenoviruses expressing p53, MMAC, β-galactosidase, and empty virus. We show that *in vitro* proliferation of human and mouse tumor cells expressing high levels of activated/phosphorylated Akt was inhibited by both Akt-DN and p53, in comparison with control viruses expressing β-galactosidase. Similarly, Akt-DN mutant expression led to selective induction of apoptosis in tumor cells expressing activated Akt. On the other hand, Akt-DN expression had minimal effect in normal and tumor cells expressing low levels of activated Akt. Expression of MMAC induced selective apoptosis in tumor cell lines in which MMAC is inactivated but not in tumor cells expressing wild-type levels of MMAC. In addition, the growth of tumor cells in a mouse model was also significantly inhibited by intratumoral injection of Akt-DN virus. These studies validate the usefulness of targeting Akt for new drug discovery efforts and suggest that inhibition of Akt may have a selective antitumor effect.

INTRODUCTION

Akt, also known as PKB,[2] is the cellular homologue of the viral oncogene v-*Akt* of the transforming murine leukemia virus Akt-8 (1). Akt/PKB is an important regulator of cell survival, cell proliferation, and insulin signaling (2–6). There are three mammalian isoforms of this serine/threonine kinase, Akt1, Akt2, and Akt3. Each isoform is composed of three distinct and conserved domains: an NH_2-terminal pleckstrin homology domain, a catalytic protein kinase domain and a short COOH-terminal tail region. Akt is activated as a consequence of agonist-induced stimulation of PI-3 kinase and generation of the phospholipid PIP_3. This second messenger interacts with the pleckstrin homology domain of Akt, recruiting the kinase to the plasma membrane and exposing threonine (Thr^{308}) and serine (Ser^{473}) residues for phosphorylation by membrane-associated protein kinases (7, 8). The pathway leading to Akt activation can be antagonized by a phosphatase that removes the 3′-phosphate from phosphatidylinositol 3,4-bisphosphate and PIP_3 (9). Recent studies have shown that this lipid phosphatase is a tumor suppressor called MMAC or PTEN that is deleted or mutated in several cancers including mammary, prostate, and brain carcinomas (2, 10–12). Because PI lipids containing 3′-phosphates are primary activators of Akt, the loss of MMAC in tumors results in high basal activity of Akt, and reintroduction of MMAC suppresses Akt activity. Hence, the suppression of tumor growth by MMAC appears to be mediated via its ability to regulate Akt enzymatic activity (13).

Akt phosphorylates a number of proteins involved in apoptosis, cell cycle regulation, protein synthesis, and glycogen metabolism (5, 6). Akt exerts antiapoptotic effects through phosphorylation of substrates that directly regulate the apoptotic machinery such as Bad and procaspase-9 (14, 15). Akt also phosphorylates proteins that indirectly inhibit apoptosis such as forkhead transcription factors, IκB kinase and mdm-2 (16–18). Phosphorylation of forkhead transcription factors by Akt prevents their nuclear localization and repression of proapoptic genes such as *BIM*, *FasL*, and TGF-β2 (19–21). In addition, Akt phosphorylates mdm-2, thus promoting its translocation to the nucleus where it can bind to p53 and target it for degradation (18). Akt stimulates cell cycle progression by phosphorylation of the cyclin-dependent kinase inhibitors p27 and $p21^{WAF1}$ causing their retention in the cytoplasm and loss of growth-inhibitory effects (22, 23). In addition, Akt affects cell proliferation through signals to the cell cycle machinery, specifically inducing accumulation of cyclin D1 levels via inactivation of glycogen synthase kinase-3 and expression of cyclin-dependent kinase inhibitors p27 and $p21^{WAF1}$ (22, 24, 25).

Amplifications of genes encoding Akt isoforms as well as its upstream regulator PI-3 kinase have been found in many tumors (2). Moreover, the finding that MMAC, one of the most frequently mutated tumor suppressor genes in human cancer, encodes a PIP_3 lipid phosphatase, provided compelling evidence linking Akt to oncogenesis (26). In addititon, Akt is a downstream target of many receptor-stimulated pathways involved in breast cancer including estrogen receptor α, epidermal growth factor receptor, and erbB2 (27, 28). Dominant negative alleles of Akt have been shown to block survival that is mediated by insulin-like growth factor 1, and constitutively active Akt rescues MMAC-mediated apoptosis (11, 29). Because Akt promotes both cell survival and proliferation, specific inhibition of its activity is a rational therapeutic strategy for tumors with amplifications of Akt or PI-3 kinase or mutations in MMAC. However, proof-of-principle that blocking Akt activity leads to inhibition of tumor cell growth *in vitro* and *in vivo* is lacking. In addition, it has not been clearly established whether blocking Akt activity sensitizes tumor cells to undergo apoptosis through the action of other stimuli or induces a direct apoptotic response selectively in tumor cells but not

Received 3/18/03; revised 6/30/03; accepted 7/25/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Tumor Biology, K-15-4 (4600), Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033. Phone: (908) 740-7328; Fax: (908) 740-3918; E-mail: Chandra.kumar@spcorp.com.

[2] The abbreviations used are: PKB, protein kinase B; PI-3 kinase, phosphatidylinositol-3 kinase; MMAC, mutated in multiple advanced cancers suppressor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; Akt-DN, Akt-dominant negative; NHDF, normal human dermal fibroblast cells; HA, hemagglutinin antigen; Ad-, adenovirus-; mTOR, mammalian target of rapamycin; CMV, cytomegalovirus; -WT, -wild type; AD5, adenovirus 5; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β-gal, β-galactosidase; FACS, fluorescence-activated cell sorter; MEK, mitogen-activated protein kinase kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate.

in normal cells. Hence, we undertook a systematic approach to inhibit Akt signaling in tumor and normal cells and to examine its consequences on cell proliferation, apoptosis, and growth in mouse xenograft models. A kinase-dead mutant of Akt was used as a dominant negative mutant. In this mutant, both Thr^{308} and Ser^{473}, which are critical for Akt activation, have been changed to alanine. The Akt-DN mutant was introduced into several tumor and normal cells using a replication-defective adenoviral expression system. Our results show that expression of Akt-DN inhibits cell proliferation and induces an apoptotic response selectively in tumor cells expressing activated Akt but not in normal cells. In addition, we show that the intratumoral injection of Akt-DN virus abolishes tumor growth in a xenograft mouse model.

MATERIALS AND METHODS

Cell Culture. MidT cells have been described previously (30). HEK-293, DU145, A549, LNCaP, MDA-MB-231, MDA-MB-453, IMR-90, ZR75-1, and CCD112CoN were obtained from American Type Culture Collection (Manassas, VA). NHDF was obtained from BioWhittaker, Inc. (Walkersville, MD) and cultured as recommended by the supplier. DU145, A549, and HEK-293 cells were cultured in DMEM. MDA-MB-231, LNCaP, and ZR75-1 were cultured in RPMI 1640. IMR-90 and CCD112CoN were maintained in MEM. MDA-MB-453 cells were cultured in Leibovitz's L-15 medium. All culture media were supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 20 units/ml penicillin, 20 μg/ml streptomycin, and 0.1 mM nonessential amino acids. Media and supplements were purchased from Invitrogen (Carlsbad, CA). All cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C, except MDA-MB-453 which were maintained in 0% CO_2.

Plasmid Construction. HA-tagged kinase-dead mutant of Akt1 with point mutations at T308A and S473A in pWeezle retroviral vector was obtained from Dr. Richard Roth (Stanford University School of Medicine). Akt-DN-HA was isolated with *Sal*I/*Bam*HI digestion and cloned into pcDNA3.1$^+$ vector (Invitrogen). Human Akt1 was amplified from a marathon-ready human lung cDNA library (Clontech, Palo Alto, CA) as described previously (31) and cloned into pcDNA3.1/*myc*-HisA (Invitrogen) to generate COOH-terminal *myc*-tagged wild-type Akt1.

Virus Construction and Purification. Ad-p53, Ad-MMAC, and Ad-β-gal are E1/E3-deleted adenovirus vectors that contain an expression cassette, cloned in the E1-deletion, in which transgene expression is driven by the CMV promoter (32, 33). The Ad-Akt-DN E1/E3-deleted adenovirus vector was constructed by cloning the Akt-DN sequences (see above) in an adenovirus E1 transfer plasmid containing an expression cassette with the CMV promoter and SV40 poly(A) addition site. Homologous recombination in *Escherichia coli* strain BJ5183 using the method of Chartier *et al.* (34) was then used to generate infectious viral DNA which was subsequently transfected into HEK-293 cells to generate virus. The Ad5-based viral backbone used for the constructs contains deletions in the E1 region (Ad5 coordinates 459-3327) and in the E3 region (Ad5 coordinates 25838–32004). Viruses were propagated in the HEK-293 cell line, purified by column chromatography (35), quantitated, and dosed by particle yield based on guidance from the Food and Drug Administration for dosing of adenovirus (Guidance for Human Somatic Cell Therapy and Gene Therapy, Center for Biologics Evaluation and Research, March 1998).

Cell Viability Assay. Cells were plated (2000 cells/well) in 96-well microtiter plates. The following day, different concentrations of adenoviruses in tissue culture media were added to the cells. Seventy-two hours later, proliferation was quantitated using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, MTS tetrazolium and phenazine methosulfate (20:1) were added to the cells. MTS is bioreduced by metabolically active cells into formazan. After 3 h at 37°C, the quantity of formazan product was measured by the amount of absorbance at 490 nm on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Each data point is the average absorbance reading of four wells, and each cell line was tested at least twice.

Apoptosis Assays. For FACS, cells were plated (2×10^5 cells/well) in six-well plates. The following day, adenoviruses in 1 ml of tissue culture media were added to the cells (final concentration, 1×10^9 particles/ml). After 6 h, virus was removed, and fresh medium was added to cells. Forty-eight hours later, both adherent and floating cells were harvested and stained with annexin V-FITC (100 ng) and propidium iodide (500 ng; Clontech) for 15 min in the dark at room temperature followed by flow cytometric analysis. For ELISAs, cells were plated (2000 cells/well) in 96-well plates. The following day, different concentrations of adenoviruses in tissue culture media were added to the cells. Apoptosis was analyzed 48–96 h later using the Cell Death Detection $ELISA^{PLUS}$ kit (Roche). Each data point is the average absorbance reading of two wells, and each cell line was tested at least twice.

Western Blotting. Cells were harvested and lysed as described above. Samples were electrophoresed on 10% or 12% 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol-NuPAGE polyacrylamide gels (Invitrogen), transferred to 0.45-μm nitrocellulose membranes (Invitrogen), and probed with antibody. Total Akt and phospho-Akt Ser^{473} antibody were obtained from Cell Signaling Technology (CST, Beverly, MA). HA antibody (clone 12CA5) was obtained from Roche. GAPDH antibody was obtained from Research Diagnostics, Inc. (Flanders, NJ). The protein/antibody complex was visualized using either an antimouse (Amersham, Piscataway, NJ) or antirabbit (Cell Signaling Technology) IgG horseradish peroxidase-linked antibody and the ECL Western blotting detection system (Amersham).

***In Vivo* Tumor Model.** BALB/Cc-*nu/nu* (nude) mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). ZR75-1 breast tumor cells were concentrated to 5×10^6/200 μl and injected into the left flank. Tumors were allowed to develop to a size of 50 mm^3 and distributed into groups of 5. Adenovirus treatment was for 5 consecutive days at 1×10^{10}/dose. Virus solution (25 μl) was injected into each of four quadrants of the tumors (total volume, 100 μl). Animals were observed daily and tumors were measured weekly. For statistical analysis, one-way ANOVA was performed on the tumor sizes on the last day (day 93) of the study and additionally analyzed using Tukey's posttest.

RESULTS

Characterization and Expression of an Akt Kinase-dead Mutant in an Adenoviral Expression System. The recombinant adenoviral vectors used in this study are shown in Fig. 1. Each contains a common vector backbone and an expression cassette in which the transgene is expressed from the constitutively active CMV immediate early promoter. Ad-Akt-DN expresses an Akt kinase-dead mutant protein in which both Thr^{308} and Ser^{473} are changed to alanine. A number of studies have shown that this double mutant Akt exerts a dominant negative effect on endogenous Akt activity in several cell types (36–38). Ad-MMAC encodes the MMAC/PTEN tumor suppressor and has been shown to induce either cell cycle arrest or

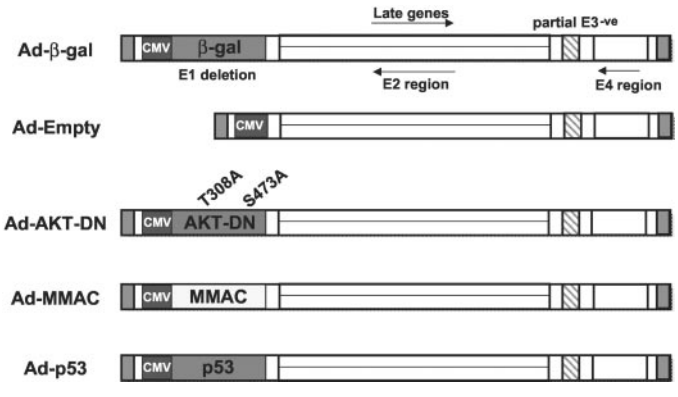

Fig. 1. Adenovirus constructs used in this study. Schematic illustrations of the standard first generation replication-defective vectors, with early region 1 (*E1*) and early region 3 (*E3*) mutations and expression cassettes with the CMV early promoter (*CMV*) driving expression of β-gal, AKT-DN, p53, or MMAC, as indicated (for more details on the virus constructs, see "Materials and Methods"). Ad-empty contains the CMV promoter but does not contain a transgene. In the AKT-DN mutant, both Thr^{308} and Ser^{473} are changed to alanine.

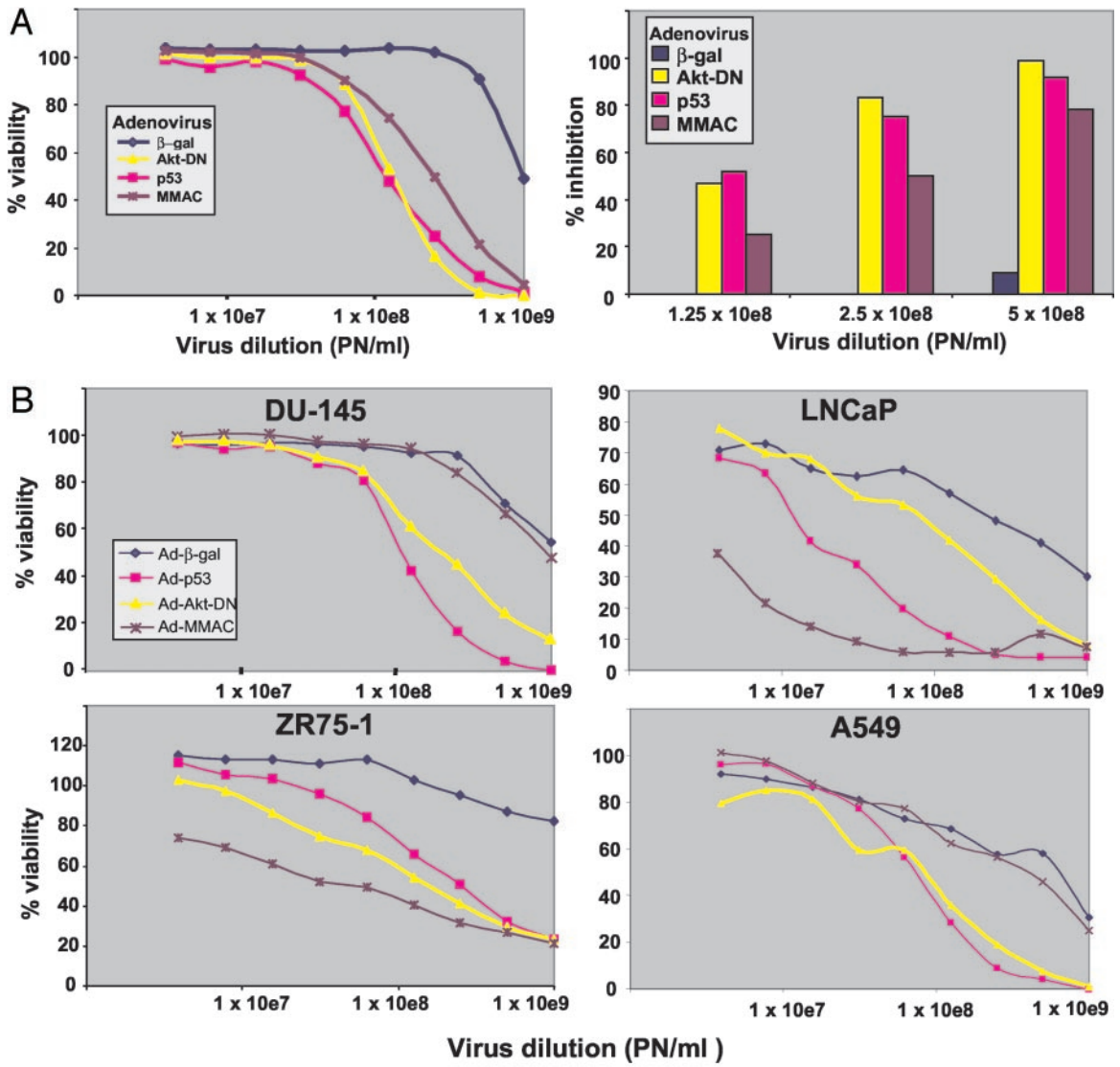

Fig. 2. *A*, Ad-Akt dominant negative mutant inhibits the growth of several tumor cells. MidT cells (2000/well) plated in 96-well microtiter plates were infected with different adenoviruses at various dilutions, and 72 h later viable cells were quantitated by adding MTS reagent and reading absorbance at 490 nm. *B*, Ad-Akt-DN inhibits the growth of DU145 and LNCaP (prostate tumor), ZR75-1 (breast tumor), and A549 (lung cancer). Human tumor cells (2000/well) plated in 96-well microtiter plates were infected with different adenoviruses at various dilutions, and 72 h later viable cells were quantitated by adding MTS reagent and reading absorbance at 490 nm. Each data point is the average absorbance reading of four wells, and each cell line was tested at least twice. Results of a representative experiment are shown. *PN*, particles; *e*, exponent (*e.g.* 10e8 = 10^8).

apoptosis in different cell types (33, 39). Ad-p53 virus expressing wild-type p53 was used as a positive control because of its established role in inducing apoptosis in several cell types (40, 41). Ad-β-gal, expressing β-galactosidase, and Ad-empty, lacking a transgene, were used as negative controls.

The kinase-dead status of the Akt-DN mutant used to construct Ad-Akt-DN virus was confirmed in preliminary studies. HEK-293 cells were transiently transfected with mammalian expression vectors encoding *myc*-tagged Akt-WT or HA-tagged Akt double mutant. Two days after transfection, cells were serum starved overnight and then stimulated with insulin (100 nM) for 20 min. Transfected Akt proteins were immunoprecipitated from equal amounts of total cell lysates using antibodies against the tag epitopes and assayed for kinase activity using an Akt-specific substrate peptide. Results confirmed that the immunoprecipitated Akt-WT protein phosphorylates the peptide substrate and its activity is stimulated by insulin treatment, whereas the Akt-DN mutant is kinase-dead and is not activated by insulin treatment (results not shown). To further characterize the expression of the Akt-DN protein from the adenoviral vector system, cell lysates from Akt-DN virus infected HEK-293 cells were analyzed by Western blot analysis using antibodies specific for the HA epitope, total Akt and phospho-specific (Thr^{308} and Ser^{473}) Akt antibodies. The results indicated that Akt-DN protein can be detected as early as 16 h after infection and confirmed high level expression of the mutant Akt protein that is recognized by total Akt antibody, but not by phospho-specific (Thr^{308}) Akt antibody (results not shown).

Akt Kinase-dead Mutant Inhibits the Growth of Tumor Cells. To determine whether the Akt kinase-dead mutant had any effect on the growth of tumor cells expressing activated Akt, MidT tumor cells derived from mammary tumors from transgenic mice expressing the polyoma middle T antigen were chosen for analysis. This cell line expresses high levels of activated/phosphorylated Akt and is sensitive to growth inhibition after infection with Ad-p53 and Ad-MMAC viruses (30). MidT cells grown in 96-well microtiter plates were infected with the recombinant adenoviruses listed in Fig. 1 at various dilutions starting with 1×10^9 particles/ml. Three days after infection, the viable cells were quantitated by a cell viability assay as described in "Materials and Methods." The results, shown in Fig. 2*A*, indicate

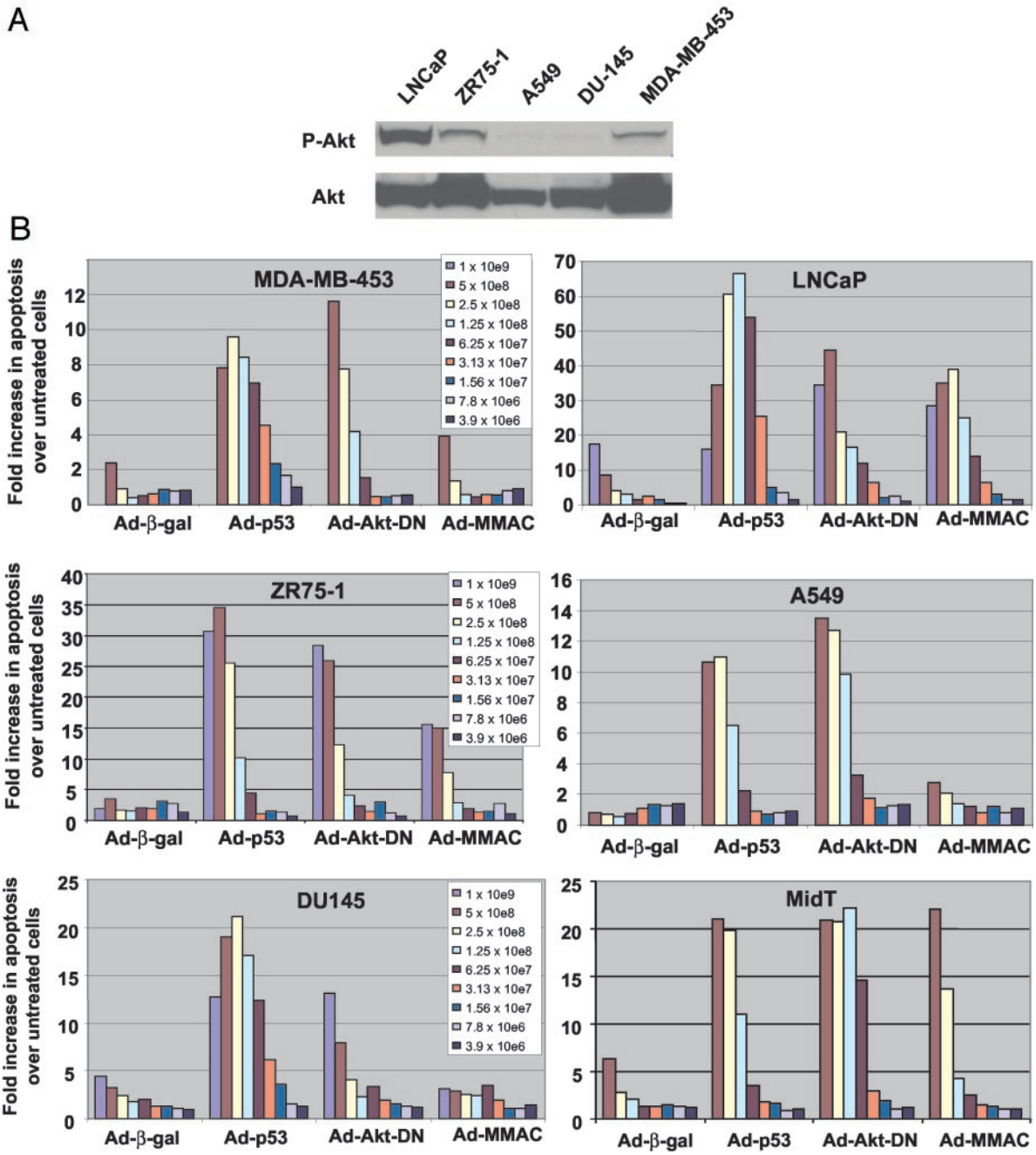

Fig. 3. Ad-Akt-DN mutant induces apoptosis in several tumor cell lines. *A*, Western blot analysis of five tumor cell lines using total and phospho-specific (Ser^{473}) Akt antibodies. In *B*, tumor cells plated in 96-well microtiter plates were infected with various adenoviruses at different concentrations. After 48 h, cytoplasmic fractions prepared from cell lysates were used for quantitation of nucleosomes using the Cell Death ELISA kit. *C*, dominant negative Akt induces apoptosis in DU145 tumor cell-FACS analysis. DU145 cells plated in six-well plates (2×10^5 cells/well) were infected with various adenoviruses at 1×10^9 particles/ml. After48 h, cells were harvested and stained with annexin V-FITC and propidium iodide (*PI*; Clontech) followed by flow cytometric analysis. Total percentage of annexin V-positive cells is shown at the top of each diagram. Each cell line was tested at least twice, and each data point is the average absorbance reading of two wells. Results of a representative experiment are shown; *e*, exponent (*e.g.* 10e8 = 10^8).

that MidT cell growth was significantly attenuated after infection with Ad-Akt-DN, Ad-p53, and Ad-MMAC viruses over many viral doses but not with Ad-β-gal virus. At high viral concentrations (1×10^9 particles/ml), a nonspecific inhibitory effect predominated as has been noted in several cell lines (33, 42). The percentages of growth inhibitions induced by different adenoviruses at three virus dilutions are shown in Fig. 2*A*, *right*. The percentages of growth inhibitions observed for Ad-Akt-DN, Ad-p53, and Ad-MMAC were 83, 75, and 50%, respectively, at the viral dose of 2.5×10^8 particles/ml (Fig. 2*A*). We have extended this type of analysis to several human tumor cell lines such as DU145 and LNCaP (prostate tumor cells), ZR75-1 (breast) and A549 (non-small cell lung cancer). DU145 and A549 tumor cells express wild-type MMAC protein, whereas ZR75-1 and LNCaP cell lines express mutant MMAC protein (21, 43). The results shown in Fig. 2*B* indicate that Akt-DN mutant inhibits the growth of all four human tumor cells in a manner similar to that of p53 virus, whereas Ad-MMAC virus selectively inhibited the growth of ZR75-1 and LNCaP tumor cells that are mutant for MMAC protein.

Akt Kinase-dead Mutant Induces Apoptosis in Tumor Cells. To investigate whether the decrease in tumor cell viability seen after Ad-Akt-DN infection correlated with an increase in apoptosis, we quantitated apoptosis using the Cell Death Detection ELISA^{Plus} kit

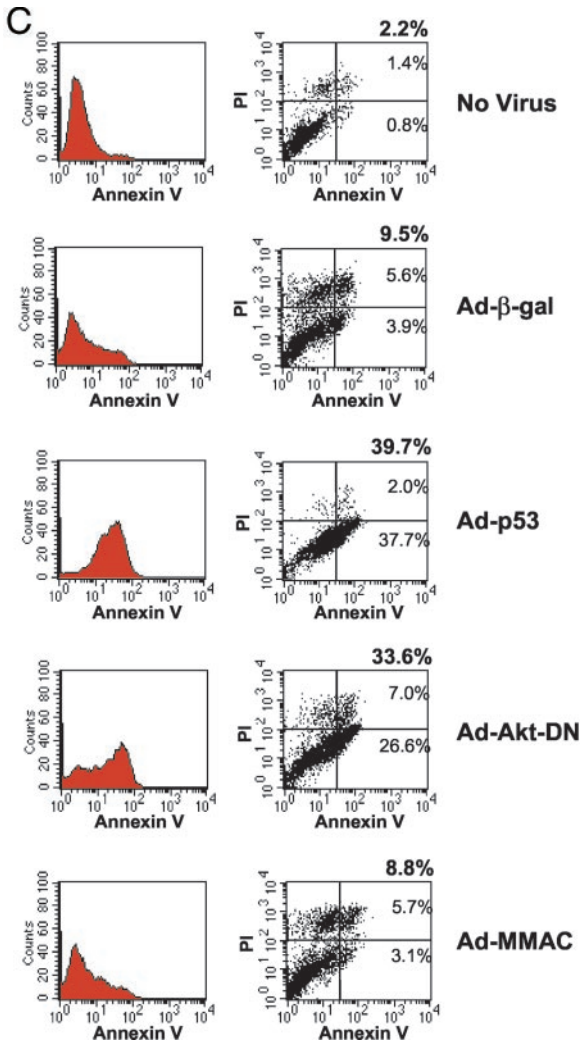

Fig. 3. *Continued.*

(Roche). This method uses antibodies directed against DNA and histones for quantitation of mono- and oligonucleosomes in the cytoplasm of cells undergoing apoptosis. We selected six tumor cell lines, MDA-MB-453, ZR75-1, and MidT (breast), LNCaP and DU145 (prostate), and A549 (lung cancer) for analysis. The MDA-MB-453 cell line, in which the *Her2/neu* gene is amplified, expresses wild-type MMAC protein similar to those in A549 and DU145 cell lines (43, 44). Western blot analysis of these cells using phospho(Ser^{473})-specific Akt antibodies shown in Fig. 3*A* indicate that all these cell lines express Ser^{473}-phosphorylated Akt protein at different levels. DU145 and A549 tumor cells express relatively low levels of Ser^{473}-phosphorylated Akt compared with LNCaP, ZR75-1, and MDA-MB-453 cells.

To quantitate apoptosis, tumor cells were plated in microtiter plates and infected with different adenoviruses at various dilutions; 48 h later, cell lysates were analyzed using the cell death ELISA method. The fold increase in apoptosis in virus-infected cells, compared with uninfected cell controls, was plotted for various virus dilutions. Fig. 3*B* shows the results for MDA-MB-453, MidT, and ZR75-1 (breast), LNCaP and DU145 (prostate), and A549 (lung cancer) tumor cell lines. All six cell lines exhibited apoptotic response after infection with Ad-p53 and Ad-Akt-DN, whereas Ad-β-gal had very little effect. These results also show that the apoptotic response is dose dependent for both Ad-Akt-DN and Ad-p53. MDA-MB-453, DU145, and A549 tumor cell lines which express wild-type MMAC protein were resistant to Ad-MMAC virus infection. In contrast, Akt-DN mutant induced apoptosis in all six cell lines irrespective of their MMAC status. In addition, Akt-DN mutant-induced apoptotic response is independent of the p53 status as DU145, ZR75-1, MDA-MB-453, and MDA-MB-231 cells express mutant p53 protein (45, 46), whereas MidT, LNCaP, and A549 cells express wild-type p53 protein (30, 47, 48).

Previous studies have shown that even though DU145 cells express wild-type MMAC protein, they are sensitive to rapamycin, a small molecule inhibitor of mTOR, a kinase that is a downstream target of the PI-3 kinase/Akt pathway (44, 49). This study concluded that DU145 cells exhibit activated Akt/mTOR signaling. Results shown in Fig. 3*B* indicate that Ad-p53 and Ad-Akt-DN elicited apoptotic response in DU145 cells, whereas infection with Ad-β-gal and Ad-MMAC had very little effect. To confirm the Akt-DN mutant-induced apoptotic response by an independent method, DU145 cells were infected with various recombinant adenoviruses and analyzed by FACS using annexin V-FITC and propidium iodide staining. DU145 cells were infected with different adenoviruses at a dilution of 1×10^9 particles/ml and both adherent and floating cells were collected 48 h later for FACS. Results shown in Fig. 3*C* indicate that infection of DU145 cells with Ad-Akt-DN and Ad-p53 resulted in a significant increase in the percentage of annexin V binding to cells from a background of 9.5 to 33.6 and 39.7%, respectively. As seen using ELISA, Ad-MMAC infection caused no significant increase in annexin V staining compared with background, because these cells express wild-type MMAC protein. Together, these results show that even though DU145 cells express low levels of activated Akt, Ad-Akt-DN mutant was able to elicit an apoptotic response.

Akt Kinase-dead Mutant-induced Apoptosis in Tumor Cells Is Selective. In the next set of experiments, we investigated whether Akt-DN mutant induced apoptosis nonselectively in all tumor cells. Both MDA-MB-231 (breast) and Malme-3M (melanoma) are human tumor cell lines expressing low levels of activated Akt (43, 50). Western blot analysis of total cell lysates using phospho(Ser^{473})-specific Akt antibodies showed low levels of expression of activated Akt in these cells (Fig. 4*A*). Fig. 4*B* shows the results of MTS assay and cell death ELISA for these two cell lines after infection by various adenoviruses. The results indicate that both MDA-MB-231 and Malme-3M cell lines were resistant to growth inhibition and apoptotic induction by Ad-Akt-DN and Ad-MMAC, whereas Ad-p53 elicited significant apoptotic response. To assess the infectivity of MDA-MB-231 and Malme-3M cell lines by adenoviruses, Ad-β-gal-infected cells were stained for β-galactosidase expression using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. Twenty-four h after infection at a viral dose of 1×10^9 particles/ml, ~50% of the Malme-3M cells and >95% of the MDA-MB-231 cells stained positive for β-gal, suggesting that these cells are susceptible to infection by adenoviruses (results not shown). Induction of apoptosis by Ad-p53 virus in these two cell lines supports this conclusion. Together, these results show that the resistance of MDA-MB-231 and Malme-3M cell lines against apoptosis by Ad-Akt-DN and Ad-MMAC is not attributable to lack of infectivity by the adenoviruses.

Akt Kinase-dead Mutant Does Not Induce Apoptosis in Human Normal Cells. To determine whether normal human cell lines are affected by Ad-Akt-DN infection, we selected IMR-90 (normal human lung fibroblast), NHDF (normal human dermal fibroblasts), and CCD112CoN (normal colon cells) for analysis. Western blot analysis of these cells using phospho(Ser^{473})-specific Akt antibodies shown in Fig. 5*A* indicates that all these cell lines express low levels of Ser^{473}-phosphorylated Akt protein similar to that of the MDA-MB-231 cell line. As described above, cells plated on microtiter plates were infected with different adenoviruses starting from a viral dose of

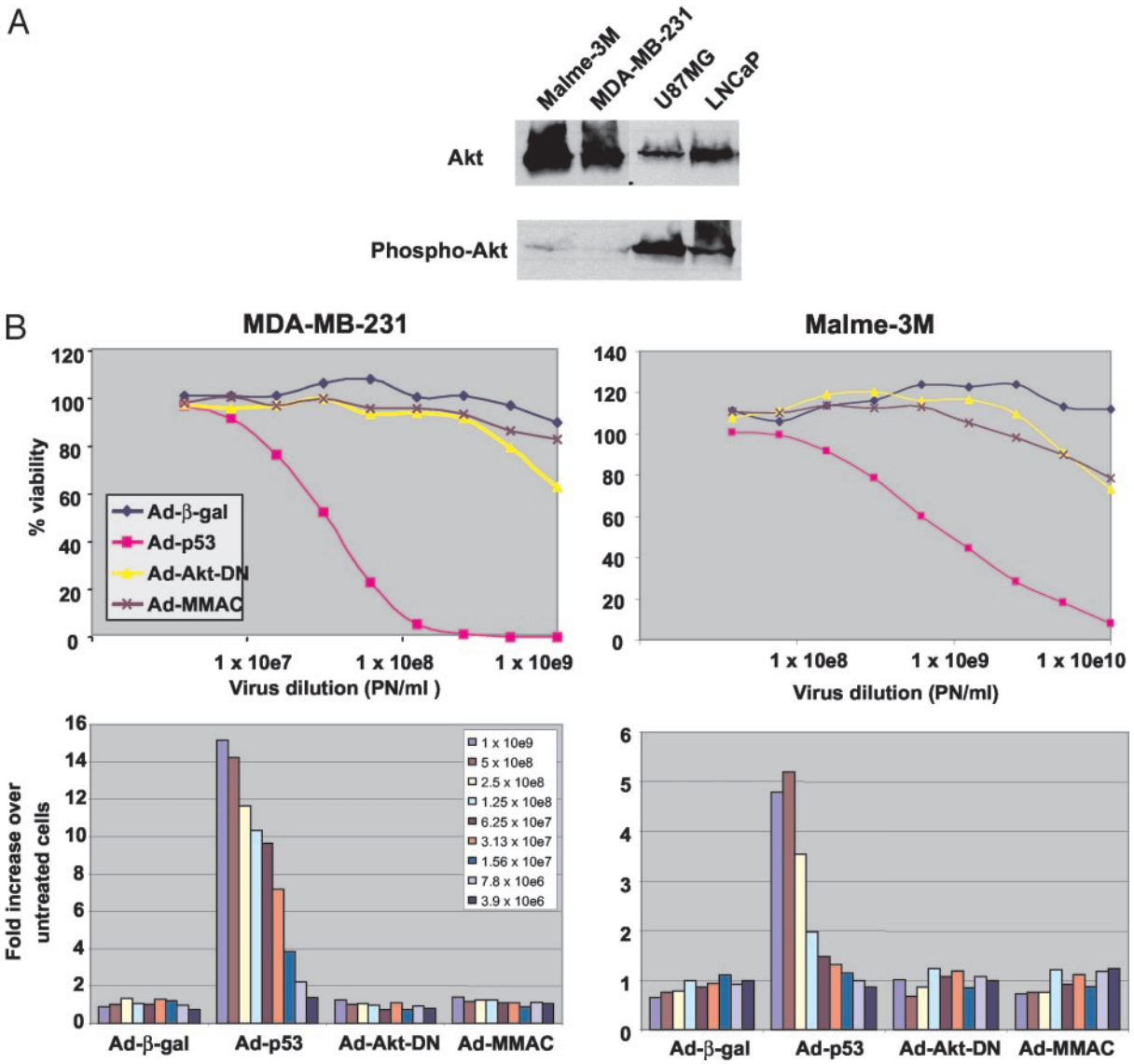

Fig. 4. Ad-Akt-DN-induced apoptotic effect is selective to tumor cells expressing activated Akt. *A*, Western blot analysis of Malme-3M, MDA-MB-231, U87MG, and LNCaP cells using total and phospho-specific (Ser^{473}) Akt antibodies. In *B*, MDA-MB-231 breast tumor cells and Malme-3M melanoma cells were infected with various recombinant adenoviruses and analyzed for viability after 72 h and for apoptosis after 48 h using the Cell Death ELISA kit. Each cell line was tested at least twice, and results of a representative experiment are shown. *PN*, particles; *e*, exponent (*e.g.* 10e8 = 10^8).

1×10^9 particles/ml, and 48–72 h later cell lysates were analyzed by cell death ELISA. Results shown in Fig. 5*B* indicate that Ad-p53 infection elicited significant apoptosis in all three cell lines, whereas Ad-β-gal, Ad-Akt-DN, and Ad-MMAC had very little effect. The fact that Ad-p53 was able to induce apoptosis in all these cells suggests that these cells are susceptible to adenoviral infections. In addition, Ad-β-gal (1×10^9 particles/ml)-infected normal cells were stained for β-gal expression using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. These results showed that ~75% of the NHDF cells and >95% of the IMR-90 and CCD112CoN cells stained positive for β-gal after 24 h after infection, suggesting that these cells are susceptible to infection by adenoviruses (data not shown). Next we wanted to examine whether infection for longer durations may be required to observe significant apoptotic effect in normal cells. NHDF and MDA-MB-231 (breast tumor cells) were infected with different adenoviruses for 96 h, and cell lysates were analyzed by cell death ELISA. Results shown in Fig. 5*C* indicate that only the Ad-p53 virus elicited significant apoptotic response in these two cell lines, whereas Ad-Akt-DN and Ad-MMAC had very little effect 96 h after infection.

To rule out the possibility that lack of apoptotic response with Akt-DN virus in these cells is attributable to lack of expression of the mutant protein, lysates of Malme-3M (melanoma), CCD112CoN (normal colon), MDA-MB-231 (breast), and IMR-90 (normal) cells, prepared after infection by the recombinant adenoviruses, were analyzed by Western blot. Total cell proteins were separated by PAGE, blotted onto nitrocellulose membrane, and probed using HA and total Akt antibodies. Results shown in Fig. 5*D* confirmed high expression levels of the Akt-DN (HA-tagged) protein in all four cell lines. Together, these results demonstrate that human normal cells and some tumor cells expressing low levels of activated Akt are resistant to apoptotic induction after infection by Ad-Akt-DN and Ad-MMAC, whereas tumor cells expressing activated Akt are sensitive to Ad-Akt-DN and undergo apoptosis.

Akt Kinase-dead Mutant Inhibits Tumor Growth in Xenograft Mouse Models. To study the effects of administering Ad-Akt-DN *in vivo*, a mouse tumor xenograft model was used. For this study, ZR75-1 breast tumor cells (5×10^6) were injected into the left flank of BALB/c (nude) mice. After the tumors developed to ~50 mm^3 (58

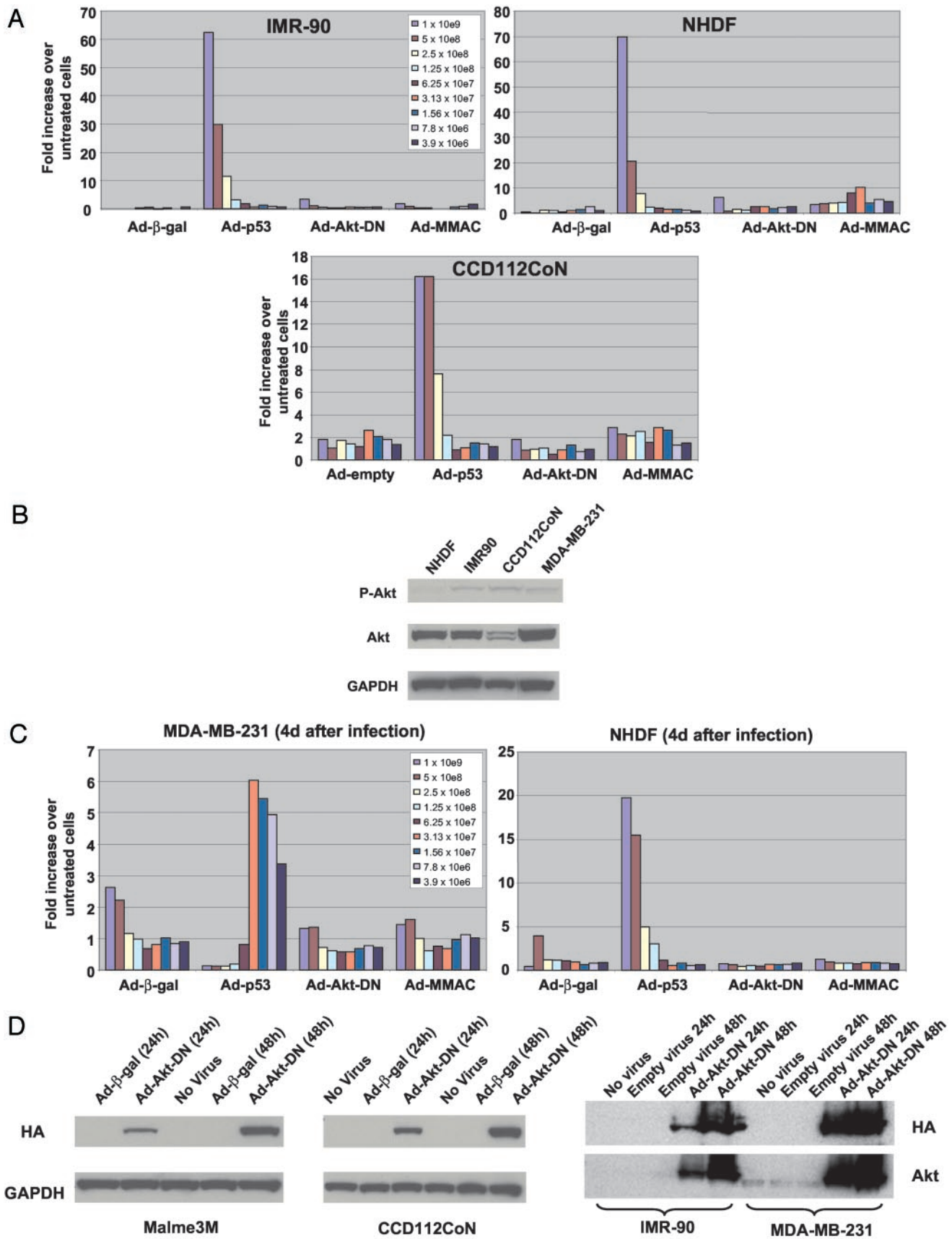

Fig. 5. Dominant negative Akt does not induce apoptosis in normal cells. *A*, IMR-90, a normal lung fibroblast cell line, and NHDF, a normal human dermal fibroblast cell line, were infected with recombinant adenoviruses and analyzed for apoptosis after 48 h using the Cell Death ELISA kit. CCD112CoN, a normal human colon cell line, was infected with adenoviruses and analyzed for apoptosis after 72 h. *B*, Western blot analysis of total cell lysates probed with Akt, phospho-Akt (Ser^{473}), and GAPDH antibodies. In *C*, MDA-MB-231 and NHDF cells were infected with various adenoviruses and analyzed for apoptosis after 96 h. *D*, expression of Akt-DN mutant protein in cells. Western blot analysis was performed on Malme-3M, CCD112CoN, IMR-90, and MDA-MB-231 cell lysates. Blots were probed with HA antibody to visualize the dominant negative protein and GAPDH antibody as a control. *4d*, 4 days.

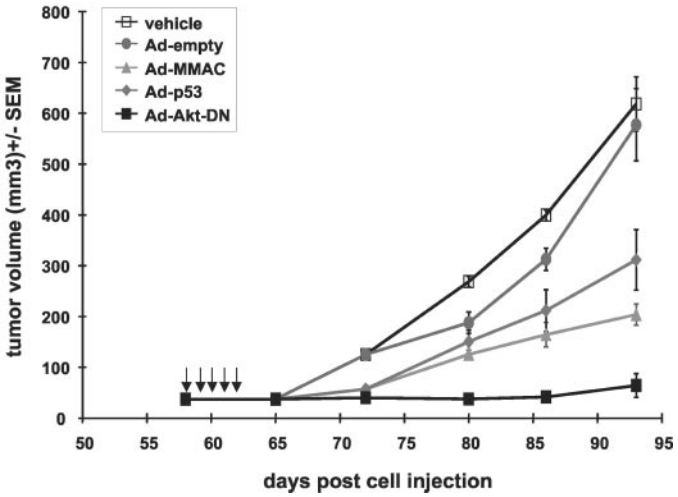

Fig. 6. Akt-DN mutant inhibits ZR75-1 tumor growth in mice. ZR75-1 tumor cells (5×10^6) were injected into the left flank of BALB/c (nude) mice. Tumors were allowed to develop to a size of 50 mm^3 (58 days) and then randomized into five treatment groups (five mice/group). Mice were dosed intratumorally once a day with 1×10^{10} particles on days 58–62. Virus solution (25 ml) was injected into each of four quadrants of the tumors.

days), the mice were randomized into five treatment groups (five mice/group) and dosed intratumorally once a day with 1×10^{10} adenovirus particles on days 58–62. Results shown in Fig. 6 indicate that although Ad-p53 and Ad-MMAC attenuated tumor growth 50 and 67%, respectively ($P < 0.001$), when compared with vehicle and Ad-empty controls, Ad-Akt-DN virus elicited the greatest effect in that tumor growth was inhibited 90% ($P < 0.001$).

DISCUSSION

Studies in the past 10 years have established Akt as an important regulator of cell proliferation and survival (2, 3). Tumor cells have devised several mechanisms to inhibit apoptosis and prolong their survival. Akt functions in an antiapoptotic pathway, and constitutively active Akt rescues cells from death induced by several stimuli. In this study, a recombinant adenoviral gene transfer system was used to demonstrate that expression of a kinase-dead mutant of Akt leads to inhibition of tumor cell growth in culture and in a xenograft mouse model. In addition, we have demonstrated that Akt kinase-dead mutant induces apoptosis selectively in tumor cells expressing activated Akt but not in normal cells. Prolonged expression of Akt kinase-dead protein had minimal effect on normal cells and in some tumor cells that express low levels of activated Akt. These results are in agreement with studies by Liu *et al.* (51), who have shown that down-regulation of Akt1 protein by antisense oligos induced apoptosis selectively in tumor cells but not in normal cells. In normal cells, Akt is believed to be present in an inactive state in the cytoplasm and is activated transiently after stimulation by growth factors or survival factors (52, 53). For example, Haas-Kogan *et al.* (52) reported that the endogenous Akt activity in several glioblastoma cells was elevated compared with human astrocytes, the normal cells from which glioblastomas are derived. Similarly, phosphorylation of Akt is elevated in several ovarian cancer cell lines compared with normal ovarian surface epithelial cells (54). Thus, Akt is a critical cell survival factor that seems to be activated in tumors by multiple mechanisms (2, 55). Studies have also shown that tumor cells maintain Akt activity under conditions of stress such as serum deprivation or detachment from matrix, whereas normal cells do not (53). Thus, tumor cells may have a dependence on Akt activity that normal cells do not share. This dependence may be even greater *in vivo* because of conditions to which tumor cells are exposed such as hypoxia, acidity, abnormal vascularization, and aneuploidy.

Recent studies by Brognard *et al.* (27) and Clark *et al.* (43) have shown that PI-3 kinase inhibitors such as LY294002 and wortmannin inhibited Akt phosphorylation and increased apoptosis in tumor cells with high levels of activated Akt but not in tumor cells with low levels of activated Akt. Studies by Dhawan *et al.* (56) have also shown, using transient transfections, that melanoma cells expressing constitutively active Akt undergo apoptosis after expression of a kinase-dead mutant of Akt, whereas cells that exhibited low levels of phosphorylated Akt were resistant. DU145 and A549 tumor cells are an exception to this correlation in that they seem to express relatively low levels of activated Akt but undergo apoptosis after expression of the Akt-DN mutant. On the other hand, both MDA-MB-231 and Malme-3M tumor cells express low levels of activated Akt and are resistant to both Ad-Akt-DN and Ad-MMAC virus infection. Other studies have shown that DU145 cells exhibit hyperactivation of the PI-3 kinase/Akt pathway and constitutive phosphorylation of mTOR which is a downstream target of Akt (57). Supporting evidence for the activation of the Akt pathway comes from the observation that the growth of these cells is strongly inhibited by rapamycin, an inhibitor of mTOR kinase (44, 49). The Western blot technique used in this study to monitor expression of activated Akt is not a quantitative method to assess activation of the Akt pathway. There may be quantitative differences in Akt activation in DU145 and A549 cells relative to MDA-MB-231 and Malme-3M cells that is not reflected in the Western blot analysis. Alternatively, there may be additional mechanisms independent of the Akt pathway that also affect sensitivity to Akt-DN mutant. In support of this, studies have shown that tumor cells such as MCF-7 and T47D which express wild-type MMAC are suppressed by Ad-MMAC virus, whereas other tumor cells such as DU145, A549, and glial cells containing wild-type MMAC are resistant to the suppressive effects of exogenous MMAC (Fig. 3*B*) (11, 58). Similarly, reintroduction of MMAC into different tumor cell lines that are mutant for MMAC has been shown to result in either cell cycle arrest (glioblastoma, renal carcinoma cell lines) or apoptosis (breast and prostate cancer cell lines) (39). Clearly, more studies are required to understand the basis for the selective induction of apoptosis by the Akt-DN mutant and MMAC.

Activity-deficient mutants of protein kinases in which phosphorylation sites targeted by extracellular stimuli have been replaced by neutral amino acids have been shown to act in a dominant negative manner. For example, mutants of mitogen-activated protein kinase and MEK in which regulatory phosphorylation sites have been replaced by alanine act in a dominant negative manner (59, 60). MEK-DN mutant showed increased binding to Raf, a kinase upstream of MEK, compared with wild-type MEK (61). Overexpression of a kinase-dead mutant of Akt can potentially block endogenous Akt activity by binding to the inositol lipids and to upstream protein kinases such as phospholipid-dependent kinase 1 that are necessary for its activation. It can also bind, nonproductively, to the substrates of Akt such as glycogen synthase kinase-3β, Bad, procaspase-9, etc., and prevent their phosphorylation by endogenous Akt. Akt kinase-dead mutant in an adenoviral expression system has been shown to function in a dominant negative manner in several cell types (62–64). Studies have shown that infection of Chinese hamster ovary and 3T3-L1 adipocytes with Ad-Akt-DN resulted in significant inhibition of insulin-stimulated activation of endogenous Akt (36) with very little effect on mitogen-activated protein kinase activation. Inhibition of phosphorylation of endogenous substrates of Akt such as glycogen synthase kinase-3β, Forkhead Box Class O, FOXO1, and tuberin after overexpression of kinase-dead mutants of Akt has also been demonstrated in several cell lines (38, 65). Stable expression of dominant

negative Akt2 in an ovarian cancer cell line (SKOV3) resulted in significant reduction in both tumorigenicity and metastatic spread in animals (54). Our results demonstrating that the apoptotic response induced by the kinase-dead mutant of Akt is selective to tumor cells expressing activated Akt suggest that the kinase-dead mutant is exhibiting a dominant negative effect on Akt signaling.

We believe that the approach described here using an adenoviral vector system to express a kinase-dead mutant of Akt to study the effects of a dominant negative mutant is generally applicable to other kinase targets. The development of selective protein kinase inhibitors that block or modulate diseases caused by abnormalities in signaling pathways is best exemplified by the success of STI571 (Gleevec), a specific inhibitor of Bcr-abl tyrosine kinase for the treatment of chronic myelogenous leukemia (66). To date, three protein kinase inhibitors (fasudil, rapamycin, and Gleevec) have been approved for clinical use, 23 protein kinase inhibitors are currently undergoing clinical trials, and many more are at the preclinical stage (67). In fact, protein kinases have now become the second most important group of drug targets after G-protein-coupled receptors. Proof-of-concept is critical as these efforts can pave the way for medicinal chemistry efforts to optimize leads obtained from high-throughput screening efforts, and to derive potent and selective compounds with desired pharmacokinetic properties. The approach described here using adenoviral-mediated expression of a dominant negative mutant kinase has several advantages: (*a*) recombinant adenoviruses expressing a protein of interest can be produced in large amounts rapidly; (*b*) these recombinant viruses can be used to infect a wide variety of cell lines, tumor and normal; (*c*) high-level protein expression can be achieved; (*d*) dose responses of transgene expression in cell lines can be analyzed quantitatively; and (*e*) adenoviruses can be tested in preclinical animal models. Two minor technical drawbacks of using adenoviruses for these studies include variation in infectivity of various cultured tumor cells and a variable nonspecific cytotoxic effect of adenoviruses, for which several controls must be performed.

Finally, our results suggest that tumor cells, unlike normal cells, are dependent on activated Akt for survival and are therefore more sensitive to inhibition of its activity. These studies have significant implications for preclinical and clinical development of small molecule inhibitors of Akt and suggest that if approaches to inhibit Akt/PKB by pharmacological or genetic means are developed, clinical trials should take into consideration the status of Akt activation of the tumors. This study shows that expression of Akt kinase-dead protein had minimal effect on normal cells. This specificity demonstrates that Akt inhibition may not be toxic to normal cells, an important safety consideration for the development of inhibitors of this target kinase. Finally, these studies validate the therapeutic concept that Akt inhibition elicits a selective antitumor effect and provide support for the development of small molecule inhibitors of this kinase.

ACKNOWLEDGMENTS

We thank Drs. Bill Demers, Ahmed Samatar, and Yaolin Wang for helpful discussions and Gaby Terracina, Zhuomei Lu, and Mike Malkowski for technical assistance. We gratefully acknowledge the receipt of Akt-DN mutant plasmid from Dr. Richard Roth (Stanford University School of Medicine, Stanford, CA). We thank Drs. Greg Reyes, Steve Chang, Robert Ralston, W. Robert Bishop, and Paul Kirschmeier for their support and Dr. Murali Ramachandra for his comments on the manuscript.

REFERENCES

1. Staal, S. P. Molecular cloning of the *akt* oncogene and its human homologues *AKT1* and *AKT2*: amplification of *AKT1* in a primary human gastric adenocarcinoma. Proc. Natl. Acad. Sci. USA, *84:* 5034–5037, 1987.
2. Vivanco, I., and Sawyers, C. L. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat. Rev. Cancer, *2:* 489–501, 2002.
3. Brazil, D. P., and Hemmings, B. A. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem. Sci., *26:* 657–664, 2001.
4. Lawlor, M. A., and Alessi, D. R. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J. Cell Sci., *114:* 2903–2910, 2001.
5. Datta, S. R., Brunet, A., and Greenberg, M. E. Cellular survival: a play in three Akts. Genes Dev., *13:* 2905–2927, 1999.
6. Testa, J. R., and Bellacosa, A. AKT plays a central role in tumorigenesis. Proc. Natl. Acad. Sci. USA, *98:* 10983–10985, 2001.
7. Testa, J. R., and Bellacosa, A. Membrane translocation and activation of the Akt kinase in growth factor-stimulated hematopoietic cells. Leuk. Res., *21:* 1027–1031, 1997.
8. Alessi, D. R., and Cohen, P. Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev., *8:* 55–62, 1998.
9. Maehama, T., and Dixon, J. E. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol., *9:* 125–128, 1999.
10. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., *15:* 356–362, 1997.
11. Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N., and Parsons, R. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res., *58:* 5667–5672, 1998.
12. Simpson, L., and Parsons, R. PTEN: life as a tumor suppressor. Exp. Cell Res., *264:* 29–41, 2001.
13. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc. Natl. Acad. Sci. USA, *95:* 13513–13518, 1998.
14. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, *91:* 231–241, 1997.
15. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. Regulation of cell death protease caspase-9 by phosphorylation. Science (Washington DC), *282:* 1318–1321, 1998.
16. Kops, G. J., and Burgering, B. M. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. J. Mol. Med., *77:* 656–665, 1999.
17. Romashkova, J. A., and Makarov, S. S. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature (Lond.), *401:* 86–90, 1999.
18. Mayo, L. D., and Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc. Natl. Acad. Sci. USA, *98:* 11598–11603, 2001.
19. Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., and Coffer, P. J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol., *10:* 1201–1204, 2000.
20. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, *96:* 857–868, 1999.
21. Samatar, A. A., Wang, L., Mirza, A., Koseoglu, S., Liu, S., and Kumar, C. C. Transforming growth factor-β2 is a transcriptional target for Akt/PKB via forkhead transcription factor. J. Biol. Chem., *14:* 14, 2002.
22. Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., and Hung, M. C. Cytoplasmic localization of $p21^{Cip1}$/WAF1 by Akt-induced phosphorylation in *HER-2/neu*-overexpressing cells. Nat. Cell Biol., *3:* 245–252, 2001.
23. Collado, M., Medema, R. H., Garcia-Cao, I., Dubuisson, M. L., Barradas, M., Glassford, J., Rivas, C., Burgering, B. M., Serrano, M., and Lam, E. W. Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by $p27^{Kip1}$. J. Biol. Chem., *275:* 21960–21968, 2000.
24. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature (Lond.), *378:* 785–789, 1995.
25. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. Genes Dev., *12:* 3499–3511, 1998.
26. Ali, I. U., Schriml, L. M., and Dean, M. Mutational spectra of *PTEN/MMAC1* gene: a tumor suppressor with lipid phosphatase activity. J. Natl. Cancer Inst., *91:* 1922–1932, 1999.
27. Brognard, J., Clark, A. S., Ni, Y., and Dennis, P. A. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res., *61:* 3986–3997, 2001.
28. Cuello, M., Ettenberg, S. A., Clark, A. S., Keane, M. M., Posner, R. H., Nau, M. M., Dennis, P. A., and Lipkowitz, S. Down-regulation of the *erbB-2* receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress *erbB-2*. Cancer Res., *61:* 4892–4900, 2001.
29. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science (Washington DC), *275:* 661–665, 1997.
30. Nielsen, L. L., Gurnani, M., Shi, B., Terracina, G., Johnson, R. C., Carroll, J., Mathis, J. M., and Hajian, G. Derivation and initial characterization of a mouse mammary tumor cell line carrying the polyomavirus middle T antigen: utility in the development of novel cancer therapeutics. Cancer Res., *60:* 7066–7074, 2000.

31. Kumar, C. C., Diao, R., Yin, Z., Liu, Y., Samatar, A. A., Madison, V., and Xiao, L. Expression, purification, characterization and homology modeling of active Akt/PKB, a key enzyme involved in cell survival signaling. Biochim. Biophys. Acta, *1526:* 257–268, 2001.
32. Wills, K. N., Maneval, D. C., Menzel, P., Harris, M. P., Sutjipto, S., Vaillancourt, M. T., Huang, W. M., Johnson, D. E., Anderson, S. C., Wen, S. F., Bookstein, R., Shepard, H. M., and Gregory, R. J. Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. Hum. Gene Ther., *5:* 1079–1088, 1994.
33. Cheney, I. W., Johnson, D. E., Vaillancourt, M. T., Avanzini, J., Morimoto, A., Demers, G. W., Wills, K. N., Shabram, P. W., Bolen, J. B., Tavtigian, S. V., and Bookstein, R. Suppression of tumorigenicity of glioblastoma cells by adenovirus-mediated *MMAC1/PTEN* gene transfer. Cancer Res., *58:* 2331–2334, 1998.
34. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J. Virol., *70:* 4805–4810, 1996.
35. Shabram, P. W., Giroux, D. D., Goudreau, A. M., Gregory, R. J., Horn, M. T., Huyghe, B. G., Liu, X., Nunnally, M. H., Sugarman, B. J., and Sutjipto, S. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. Hum. Gene Ther., *8:* 453–465, 1997.
36. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikkawa, U., and Kasuga, M. Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. Mol. Cell. Biol., *18:* 3708–3717, 1998.
37. Kotani, K., Ogawa, W., Hino, Y., Kitamura, T., Ueno, H., Sano, W., Sutherland, C., Granner, D. K., and Kasuga, M. Dominant negative forms of Akt (protein kinase B) and atypical protein kinase Cλ do not prevent insulin inhibition of phosphoenolpyruvate carboxykinase gene transcription. J. Biol. Chem., *274:* 21305–21312, 1999.
38. Nakae, J., Kitamura, T., Ogawa, W., Kasuga, M., and Accili, D. Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhr) requires kinases distinct from Akt. Biochemistry, *40:* 11768–11776, 2001.
39. Di Cristofano, A., and Pandolfi, P. P. The multiple roles of PTEN in tumor suppression. Cell, *100:* 387–390, 2000.
40. Atencio, I. A., Ramachandra, M., Shabram, P., and Demers, G. W. Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ., *11:* 247–253, 2000.
41. Atencio, I. A., Avanzini, J. B., Johnson, D., Neuteboom, S., Vaillancourt, M. T., Nielsen, L. L., Hajian, G., Sutjipto, S., Sugarman, B. J., Philopena, J., McAllister, D. L., Beltran, J. C., Nodelman, M., Ramachandra, M., and Wills, K. N. Enhanced apoptotic activity of a p53 variant in tumors resistant to wild-type p53 treatment. Mol. Ther., *4:* 5–12, 2001.
42. Harris, M. P., Sutjipto, S., Wills, K. N., Hancock, W., Cornell, D., Johnson, D. E., Gregory, R. J., Shepard, H. M., and Maneval, D. C. Adenovirus-mediated *p53* gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. Cancer Gene Ther., *3:* 121–130, 1996.
43. Clark, A. S., West, K., Streicher, S., and Dennis, P. A. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol. Cancer Ther., *1:* 707–717, 2002.
44. Grunwald, V., DeGraffenried, L., Russel, D., Friedrichs, W. E., Ray, R. B., and Hidalgo, M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. Cancer Res., *62:* 6141–6145, 2002.
45. Beroud, C., and Soussi, T. *p53* gene mutation: software and database. Nucleic Acids Res., *26:* 200–204, 1998.
46. Jeffy, B. D., Chirnomas, R. B., Chen, E. J., Gudas, J. M., and Romagnolo, D. F. Activation of the aromatic hydrocarbon receptor pathway is not sufficient for transcriptional repression of BRCA-1: requirements for metabolism of benzo[*a*] pyrene to 7*r*,8*t*-dihydroxy-9*t*,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Cancer Res., *62:* 113–121, 2002.
47. Colletier, P. J., Ashoori, F., Cowen, D., Meyn, R. E., Tofilon, P., Meistrich, M. E., and Pollack, A. Adenoviral-mediated *p53* transgene expression sensitizes both wild-type and null p53 prostate cancer cells *in vitro* to radiation. Int. J. Radiat. Oncol. Biol. Phys., *48:* 1507–1512, 2000.
48. Murakami, Y., and Sekiya, T. Accumulation of genetic alterations and their significance in each primary human cancer and cell line. Mutat. Res., *400:* 421–437, 1998.
49. Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H., and Sawyers, C. L. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc. Natl. Acad. Sci. USA, *98:* 10314–10319, 2001.
50. Zhou, B. P., Hu, M. C., Miller, S. A., Yu, Z., Xia, W., Lin, S. Y., and Hung, M. C. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NFκB pathway. J. Biol. Chem., *275:* 8027–8031, 2000.
51. Liu, X., Shi, Y., Han, E. K., Chen, Z., Rosenberg, S. H., Giranda, V. L., Luo, Y., and Ng, S. C. Downregulation of Akt1 inhibits anchorage-independent cell growth and induces apoptosis in cancer cells. Neoplasia, *3:* 278–286, 2001.
52. Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G., and Stokoe, D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr. Biol., *8:* 1195–1198, 1998.
53. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H. A., and Downward, J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J., *16:* 2783–2793, 1997.
54. Arboleda, M. J., Lyons, J. F., Kabbinavar, F. F., Bray, M. R., Snow, B. E., Ayala, R., Danino, M., Karlan, B. Y., and Slamon, D. J. Overexpression of AKT2/protein kinase Bβ leads to up-regulation of β_1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res., *63:* 196–206, 2003.
55. Hill, M., and Hemmings, B. Inhibition of protein kinase B/Akt. implications for cancer therapy. Pharmacol. Ther., *93:* 243–251, 2002.
56. Dhawan, P., Singh, A. B., Ellis, D. L., and Richmond, A. Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-κB and tumor progression. Cancer Res., *62:* 7335–7342, 2002.
57. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M., and Abraham, R. T. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res., *60:* 3504–3513, 2000.
58. Furnari, F. B., Lin, H., Huang, H. S., and Cavenee, W. K. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc. Natl. Acad. Sci. USA, *94:* 12479–12484, 1997.
59. Pages, G., Lenormand, P., L'Allemain, G., Chambard, J. C., Meloche, S., and Pouyssegur, J. Mitogen-activated protein kinases p42^{mapk} and p44^{mapk} are required for fibroblast proliferation. Proc. Natl. Acad. Sci. USA, *90:* 8319–8323, 1993.
60. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell, *77:* 841–852, 1994.
61. Wu, X., Noh, S. J., Zhou, G., Dixon, J. E., and Guan, K. L. Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated HeLa cells. J. Biol. Chem., *271:* 3265–3271, 1996.
62. Fujio, Y., and Walsh, K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J. Biol. Chem., *274:* 16349–16354, 1999.
63. Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., and Walsh, K. Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in mouse heart. Circulation, *101:* 660–667, 2000.
64. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature (Lond.), *399:* 597–601, 1999.
65. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell, *10:* 151–162, 2002.
66. Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat. Rev. Drug Discov., *1:* 493–502, 2002.
67. Cohen, P. Protein kinases–the major drug targets of the twenty-first century? Nat. Rev. Drug Discov., *1:* 309–315, 2002.

Adenoviral-Mediated Expression of a Kinase-Dead Mutant of Akt Induces Apoptosis Selectively in Tumor Cells and Suppresses Tumor Growth in Mice

Amanda Jetzt, John A. Howe, Mark T. Horn, et al.

Cancer Res 2003;63:6697-6706.