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Role of AKT1 in 17β-Estradiol- and Insulin-Like Growth Factor I (IGF-I)-Dependent Proliferation and Prevention of Apoptosis in MCF-7 Breast Carcinoma Cells

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ABSTRACT. AKT1 (c-AKT, PKBα) is the cellular homolog of the protein-serine/threonine kinase oncogene, v-*akt*. AKT1 is activated through the insulin and platelet-derived growth factor signaling pathways in transfected fibroblasts, but little is known about the regulation of endogenous AKT1 in tumor cells. AKT1 levels were higher in a panel of human breast carcinoma cell lines than in breast epithelial cells, particularly those with higher HER2 expression. AKT1 activity was increased by either estradiol or IGF-I in estrogen-dependent MCF-7 cells, and both factors acted synergistically to increase AKT1 activity and promote cell proliferation. Stimulation of AKT1 activity by estradiol and IGF-I was blocked by the antiestrogen ICI 182780 and by the phosphatidylinositol-3-kinase inhibitor wortmannin. MCF-7 cells transfected with AKT1 exhibited partial estrogen- and IGF-I-independent growth and were more responsive to the combination of IGF-I and estradiol. AKT1-overexpressing MCF-7 cells were less sensitive to apoptosis induced by wortmannin. These findings suggest that AKT1 is a downstream effector of estrogen- and IGF-I-dependent proliferation and survival in hormone-responsive MCF-7 breast carcinoma cells. BIOCHEM PHARMACOL **58**;3:425–430, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. AKT1; apoptosis; MCF-7 cells; IGF-I; estradiol

AKT1/PKBα is the cellular homolog of the protein-serine/threonine kinase oncogene v-*akt*, the transforming gene associated with the AKT8 retrovirus originating from the AKR mouse [1, 2]. Human AKT exists as three closely related isoforms, AKT1/PKBα, AKT2/PKBβ, and AKT2′/PKBβ′, that differ primarily in their C-terminal domains [3, 4]. All isoforms contain a unique N-terminal pleckstrin homology domain that has a high affinity for $PI(3,4)P_2$† and $PI(3,4,5)P_3$ [5]. AKT1 is activated *in vitro* by $PI(3,4)P_2$ [6] as well as in cells treated with insulin, IGF-I, and PDGF [7, 8], and lies downstream from PI3-K [9, 10]. Phosphoinositides serve to anchor the AKT signaling complex to the inner surface of the plasma membrane, thereby coupling AKT to the growth factor receptor/PI3-K complex that generates $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in the plasma membrane bilayer [11]. AKT is activated by two transphosphorylation reactions at Thr308 and Ser473 by the phosphoinositide-dependent protein kinases PDK1 and PDK2 [12–14]. AKT is also activated in a PI3-K-dependent manner by v-Ha-Ras and v-Src [9], and, therefore, both the Ras and PI3-K signaling pathways may converge at AKT.

There are relatively few studies examining either the expression or activity of AKT in cancer cells. *AKT1* is amplified in stomach cancer, and *AKT2* is amplified in breast, ovarian, and pancreatic cancer and is overexpressed in ovarian cancers in which there is no amplification of *AKT2* [15–18]. *AKT2* amplification was not related to MYC or HER2 amplification, and thus identifies an independent subset of cells whose phenotype is less differentiated and more metastatic [15, 19].

In the present study, we report for the first time that AKT1 is highly expressed in several human breast carcinoma cell lines, and that its activity in MCF-7 cells is modulated by estradiol and IGF-I. In addition, we present evidence that overexpression and activation of AKT1 in MCF-7 cells produces estrogen- and IGF-I-independent proliferation and controls an anti-apoptotic pathway.

MATERIALS AND METHODS

Reagents

Wortmannin was obtained from the Sigma Chemical Co., and human recombinant IGF-I and 17β-estradiol was

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† *Abbreviations:* FBS, fetal bovine serum; IGF-I, insulin-like growth factor-I; PDGF, platelet-derived growth factor; PI3-K, phosphatidylinositol 3-kinase; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; and RT-PCR, reverse-transcriptase-polymerase chain reaction.

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purchased from Upstate Biotechnology, Inc. All tissue culture reagents were from Life Technologies, Inc.

Cell Culture

All human breast carcinoma cell lines and MCF-10A breast epithelial cells were obtained from the Tissue Culture Core Facility, Lombardi Cancer Center, and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, and 50 μg/mL of gentamicin. Subconfluent cultures were grown for 2 days in phenol red-deficient DMEM supplemented with 5% charcoal-stripped and dextran-treated FBS. Then cells were treated for 48 hr with either 10 nM 17β-estradiol, 10 ng/mL of IGF-I, or the combination of both agents. In some experiments, cells were treated with 10 nM ICI 182780 prior to the addition of estradiol and IGF-I.

Cytotoxicity Assay

The cytotoxicity of wortmannin was determined in MCF-7 cells by clonogenic assay [20]. Cells were plated in 60-mm dishes in complete medium at a density of 200 cells per dish and were allowed to attach overnight. Cells were washed twice with medium and then treated with wortmannin. Cells were incubated for 10–15 days, and colonies were stained with Giemsa and counted [20].

DNA Fragmentation Assay

Following incubation with estradiol and IGF-I in the presence and absence of wortmannin, approximately 1×10^6 adherent and nonadherent cells were harvested, and soluble DNA was prepared. Cells were collected by centrifugation, washed twice with phosphate-buffered saline, and resuspended in 0.5 mL of lysis buffer [1% NP-40, 50 mM Tris–HCl (pH 7.5), and 20 mM EDTA]. Cells were kept on ice for 10 min, mixed gently, and centrifuged in a microcentrifuge for 10 min at 14,000 *g* at 4°; the supernatant was saved. The extraction was repeated once, the supernatants were combined, and SDS and RNase A were added to a final concentration of 1% and 0.5 mg/mL, respectively. Samples were incubated at 56° for 2 hr, proteinase K was added to a final concentration of 2 mg/mL, and the mixture was incubated at 37° for 2 hr. One-half volume of 10 M ammonium acetate and 2.5 vol. of cold ethanol were added to the supernatant and incubated overnight at −20°. DNA was collected by centrifugation at 14,000 *g* for 20 min at 4°, the DNA pellet was dissolved in 50 μL TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)] separated by electrophoresis in a 1.5% agarose gel containing 0.5 μg/mL of ethidium bromide in TAE buffer [40 mM Tris-acetate (pH 8.0) and 2 mM EDTA], and visualized by UV fluorescence.

Western Blotting and Immunocomplex Kinase Assays

Cells were scraped, washed once in phosphate-buffered saline, and centrifuged. Cells were lysed in 0.5 mL buffer containing 20 mM Tris (pH 7.6), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of pepstatin, 1 μg/mL of aprotinin, and 2 μg/mL of leupeptin. After incubation on ice for 10 min, cells were sonicated with three 10-sec bursts and centrifuged for 10 min at 14,000 *g* at 4°. Samples (50 μg protein per lane) were separated in 8% precast polyacrylamide gels (Novex, Inc.) by SDS–PAGE and transferred electrophoretically onto a nitrocellulose membrane using a semi-dry protein transfer apparatus (Schleicher & Schuell, Inc.). AKT protein was determined with a sheep AKT1 polyclonal antibody (Upstate Biotechnology, Inc.) and an ECL detection system (Boehringer–Mannheim).

Immunocomplex kinase assays were carried out with 500 μg of cell lysate that was precleared by the addition of 20 μL Protein A + G Sepharose (Oncogene Sciences, Inc.). Precleared lysates were incubated with 2.5 μL of rabbit polyclonal Akt1 antibody (provided by Dr. Alfonso Bellacosa, Fox Chase Cancer Institute) and mixed on a rocking shaker for 3 hr at 4°. The complex was incubated with 20 μL Protein A + G Sepharose and incubated for an additional hour. The complex was washed twice with lysis buffer and once in kinase equilibration buffer [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol], and kinase activity was measured by resuspending the immunocomplex in 50 μL of kinase buffer containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM ATP, 1 μM protein kinase A inhibitor peptide, 25 μg myelin basic protein, and 2 μCi [γ-^{32}P]ATP, and incubating for 30 min at 30° [7]. The reaction was stopped by the addition of 5x Laemmli sample buffer, boiled for 4 min, and then kept on ice for 10 min. Samples were separated in 12% precast gels (Novex, Inc.) by SDS–PAGE and transferred onto nitrocellulose. AKT kinase activity was quantitated by autoradiography. The same membrane was also blotted with anti-AKT polyclonal antibody to determine the amount of AKT in each lane.

Plasmid Construction and Transfection

The full-length *AKT1* cDNA was synthesized by RT-PCR using 1 μg of poly(A)RNA from MCF-7 breast cancer cells. The forward and reverse primers were 5′-GTGCATCAG-AGGCTGTGGCC-3′, and 5′-GCTATCGTCCAGCG-CAGTCC-3′, respectively. A full-length 2.2 kb fragment generated by PCR was cloned directly into plasmid pTarget (Promega) with 3′-T overhangs in its multiple cloning cassette to generate pTarget/AKT1. An *Eco*RI fragment containing the *AKT1* cDNA was excised from pTarget/AKT1, gel-purified, and subcloned into the *Eco*RI site of expression vector pcDEF-3 downstream to the human EF-1a promoter (provided by Dr. J. A. Langer, Robert

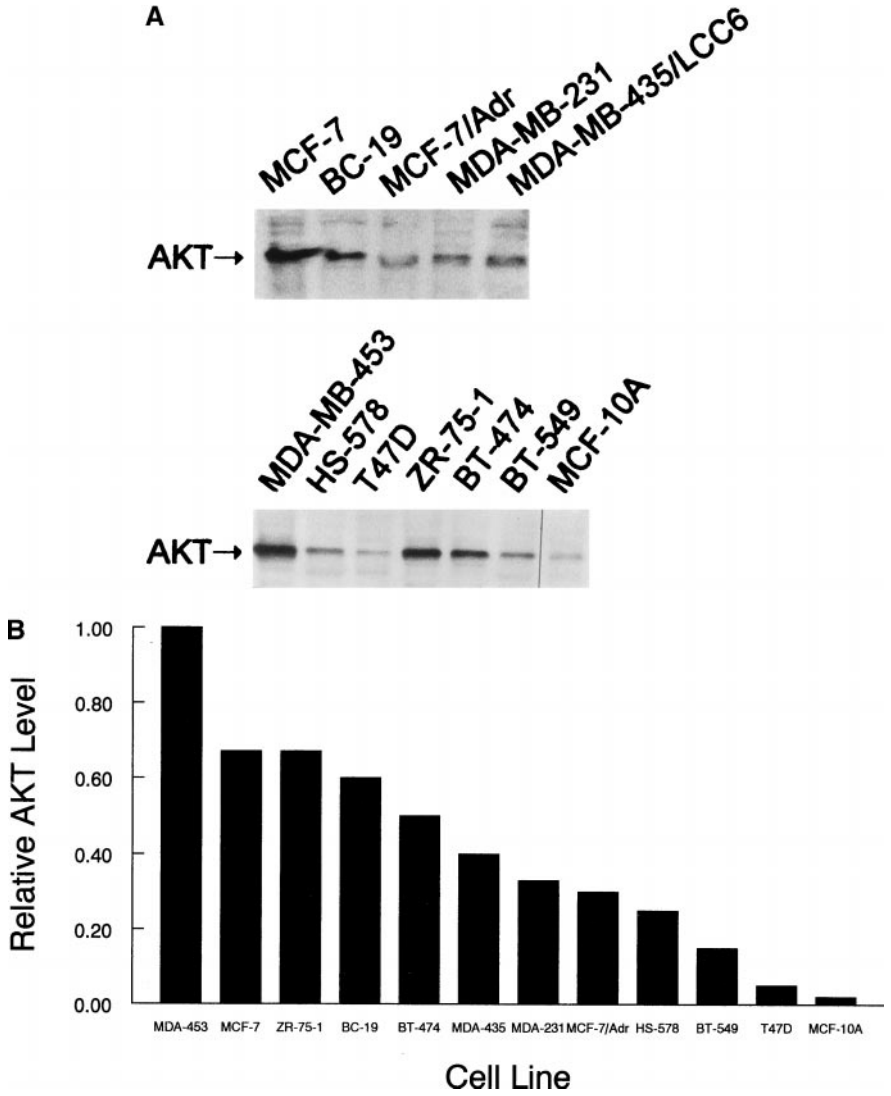

FIG. 1. AKT1 levels in human breast carcinoma and breast epithelial cells. AKT1 levels were determined by western blotting and quantitated by densitometry. Whole cell extracts (50 μg protein/lane) were separated by SDS–PAGE in an 8% gel, transferred to nitrocellulose, and probed with an isoform-specific AKT1 polyclonal antibody. (A) Western blot of AKT1 levels. (B) Quantitation of AKT1 levels by densitometry. Levels are expressed in arbitrary units relative to MDA-MB-453 cells. The levels of AKT1 relative to MDA-MB-453 are: MDA-MB-453, 1.00; MCF-7, 0.67; ZR-75-1, 0.67; BC-19, 0.60; BT-474, 0.50; MDA-MB-435, 0.40; MDA-MB-231, 0.33; MCF-7/Adr, 0.30; Hs-578, 0.25; BT-549, 0.15; T47D, 0.05; and MCF-10A, 0.02. These results are representative of two similar experiments.

Wood Johnson Medical School, Rutgers University) [21] to yield pcDEF-3/AKT1. The *AKT1* cDNA sequence was confirmed by dideoxy sequencing using an ABI sequencer (DNA Sequencing Core Facility, Lombardi Cancer Center).

The AKT1 kinase-inactive mutant, K179E, was prepared by overlap PCR. The forward and reverse primers for wild-type AKT1 encompassed nt 111-2540 in Ref. 22, and were 5′-GTGCATCAGAGGCTGTGGCC-3′ and 5′-CGCCGTGGTGCAGCGGCA-3′, respectively. The forward and reverse primers for AKT1K179E encompassed nt 727-747 and were 5′-GCCATGGAGATCCTCAAGAAG-3′ and 5′-CTTCTTGAGGATCTCCATGGC-3′, respectively (mutation underlined). After PCR amplification, AKT1K179E was subcloned into pcDEF-3, and the mutation was confirmed by sequencing.

MCF-7 cells were transfected using Lipofectin (Life Technologies, Inc.) as described previously [20], and stable transfectants were batch-selected in G418 and used without further subcloning.

RESULTS AND DISCUSSION

AKT1 is activated in fibroblasts by a number of growth factors, including PDGF, EGF and IGF-I [9, 23], but few studies have investigated its regulation and expression in tumor cells. Therefore, the level of AKT1 was determined in a panel of breast carcinoma cell lines by western blotting and densitometry using an isoform-specific antibody (Fig. 1). AKT1 expression was highest in MDA-MB-453 cells and lowest in the human breast epithelial cell line MCF-10A (Fig. 1). Although AKT1 was present in all cell lines,

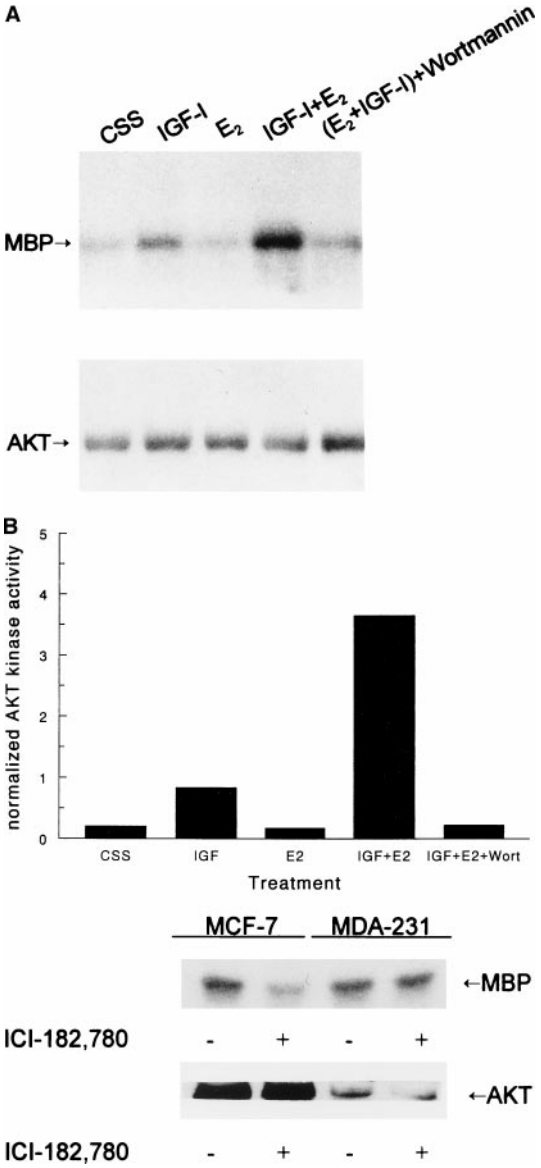

FIG. 2. AKT1 activity in MCF-7 cells treated with estradiol, IGF-I, wortmannin, and ICI 182780. (A) Immunocomplex kinase assays were performed with cell lysates (250 μg protein) prepared from MCF-7 cells grown overnight in estrogen withdrawal medium containing 5% charcoal-stripped and dextran-treated FBS (CSS), and treated with either 20 ng/mL of estradiol (E_2) or 50 ng/mL of IGF-I or a combination of both agents. Wortmannin was added at a concentration of 50 μM at the time of addition of growth factors. Upper panel: AKT1 protein kinase activity with myelin basic protein (MBP) as substrate. Lower panel: western blot of the same membrane used in the protein kinase assay to determine AKT1 levels in each lane. (B) Quantitation of AKT1 protein kinase activity shown in (A) by densitometry. AKT1 activity was normalized to AKT1 levels and expressed in arbitrary units. (C) Effect of ICI 182780 on AKT1 activity in MCF-7 cells. Immunocomplex kinase assays were carried out as described in (A) with 250 μg protein from estrogen-dependent MCF-7 cells, and 500 μg protein from estrogen-independent MDA-MB-231 cells as a negative control. Cells were treated with 10 nM ICI 182780 for 48 hr. Upper panel: AKT1 protein kinase activity with MBP as substrate. Lower panel: western blot of the same membrane used in the protein kinase assay to indicate AKT1 levels in each lane. These results are representative of two similar experiments.

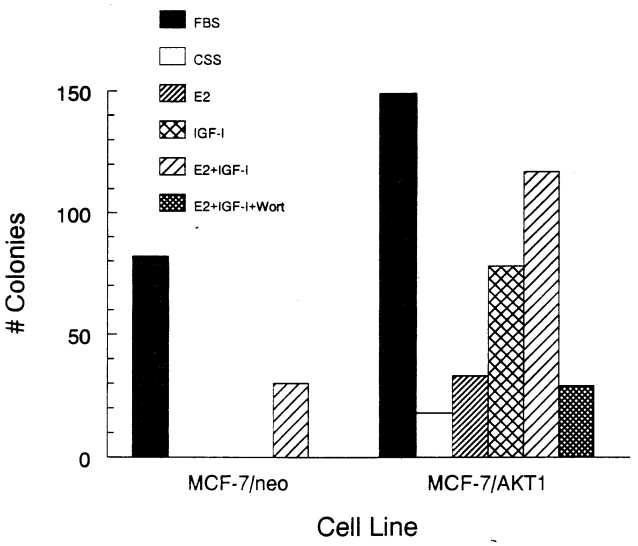

FIG. 3. Effect of estradiol (E_2) and IGF-I on the growth of MCF-7 cells transfected with AKT1. MCF-7 cells were transfected with the pcDEF-3 expression vector encoding the human *AKT1* cDNA, and batch-selected for *neo* expression with G418. Cells were grown overnight in either complete medium containing FBS (FBS) or in estrogen withdrawal medium containing 5% charcoal-stripped and dextran-treated FBS (CSS) supplemented with either 20 ng/mL of estradiol or 50 ng/mL of IGF-I, or a combination of both agents. In one instance, cells were also treated with 25 μM wortmannin (WORT) to effect apoptosis. Colony formation was determined after 2 weeks. Each value is the average of triplicate determinations.

it generally was higher in those cell lines with higher HER2 expression (MDA-MB-453, ZR-75-1, BT-474, and MCF-7) irrespective of estrogen dependence.

The proliferation of MCF-7 cells involves both hormone-dependent and -independent signaling pathways. Since estrogen sensitizes MCF-7 cells to the mitogenic effect of insulin and insulin-like growth factors [24], the effects of estrogen and IGF-I on AKT1 activity were determined by immunocomplex kinase assay using lysates from MCF-7 cells treated with estradiol and IGF-I (Fig. 2). AKT1 activity was greatly enhanced by IGF-I, but not estradiol, and both agents produced a synergistic increase in activity (Fig. 2A). Induction of AKT1 activity by estradiol and IGF-I was reduced by the PI3-K inhibitor wortmannin to pretreatment levels (Fig. 2B). The antiestrogen ICI 182780 (Fig. 2C) also reduced AKT1 activity in estrogen-dependent MCF-7 cells but not in estrogen-independent MDA-MB-231 cells (Fig. 2C).

To determine if AKT1 activity correlated with the ability of estradiol and IGF-I to promote growth and survival, MCF-7 cells were transfected with AKT1, and colony assays were performed in the presence and absence of wortmannin (Fig. 3). MCF-7/*neo* cells or wild-type MCF-7 cells (results not shown) were able to form colonies only in the presence of both estradiol and IGF-I. Overexpression of AKT1 enhanced colony formation in response to either estradiol or IGF-I, and in contrast to *neo*-expressing cells, the effect of IGF-I alone was nearly

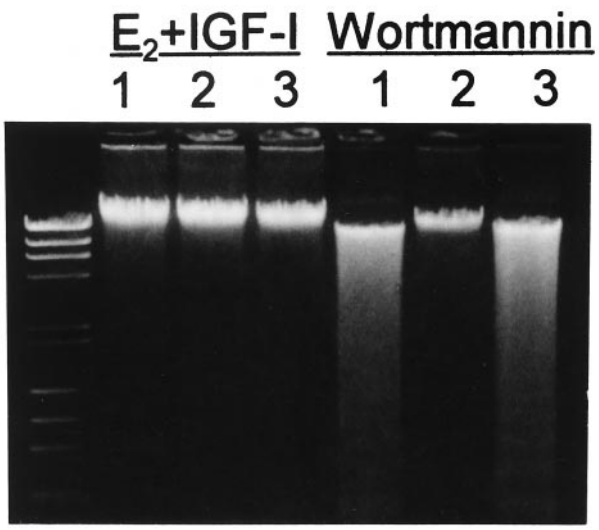

FIG. 4. Effect of AKT1 overexpression in MCF-7 cells on wortmannin-induced apoptosis. MCF-7 cells expressing either wild-type AKT1 or the kinase-inactive mutant AKT1K179E were grown in complete medium in the presence of 50 μM wortmannin. Soluble DNA was isolated and separated by electrophoresis in a 1% agarose gel, and stained with ethidium bromide. Left lane represents *Hin*dIII digested λ DNA and *Hae*III digested ϕX174 DNA as molecular weight markers. MCF-7 cells were transfected with either the empty vector, pcDEF-3 (lane 1), pcDEF-3/AKT1 (lane 2), or pcDEF-3/AKT1K179E (lane 3), and batch-selected in G418. Ethidium-stained oligonucleosomal DNA was present in pcDEF-3- and pcDEF-3/AKTK179E-transfected cells, but was not present in AKT1-transfected cells, indicating an absence of apoptosis. These results are representative of two similar experiments.

equivalent to the combination of estradiol and IGF-I. Wortmannin blocked the growth response to estradiol and IGF-I (Fig. 3) in a manner similar to its effect on AKT1 activity in untransfected cells (Fig. 2A). These results suggest that AKT1 is a common effector molecule downstream of PI3-K that links the mitogenic effects of estrogen and IGF-I.

Since estradiol and IGF-I acted synergistically to promote AKT1 activation and colony formation, we investigated whether wortmannin-mediated apoptosis could be prevented in MCF-7/AKT1 cells (Fig. 4). MCF-7 cells transfected with vectors encoding either *neo* or the AKT1-inactive K179E mutation underwent apoptosis in response to 50 μM wortmannin (Fig. 4). In contrast, MCF-7/AKT1 cells were resistant to the apoptotic effects of wortmannin. These data are consistent with previous investigations showing that expression of a constitutively active AKT1 in fibroblasts blocked apoptosis induced by wortmannin [25] and inhibited Ced3/ICE-like protease activity [26]. In these instances, the anti-apoptotic effects of AKT1 were linked to phosphorylation of the pro-apoptotic Bcl-2 family member, BAD, resulting in dissociation of the inactive heterodimeric Bcl-2:BAD complex [27].

The present study establishes that AKT1 is highly expressed in a variety of human breast carcinoma cell lines irrespective of their estrogen receptor status. AKT1 activity was enhanced in MCF-7 cells by IGF-I, and IGF-I and estradiol acted synergistically to activate AKT1 activity and proliferation. Overexpression of AKT1 in MCF-7 cells accentuated the growth effects of estradiol and IGF-I, rendering the cells partially estrogen-independent and resistant to apoptosis. These results indicate for the first time that AKT1 is a common link between the estrogen and IGF-I signaling pathways in breast cancer cells and their resistance to drug-induced apoptosis. It will be of interest in future studies to determine the anti-apoptotic pathway downstream to AKT1 in both estrogen-dependent and -independent breast cancer cells, and its relationship to drug responsiveness and invasiveness. Since AKT1 appears to be important in an anti-apoptotic pathway that controls tumor cell survival, it may also be a useful molecular target for developing antitumor therapeutics.

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