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Phosphatidylinositol 3-Kinase/AKT-mediated Activation of Estrogen Receptor α

A NEW MODEL FOR ANTI-ESTROGEN RESISTANCE*

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Estrogen receptors (ERs) mediate most of the biological effects of estrogen in mammary and uterine epithelial cells by binding to estrogen response elements in the promoter region of target genes or through protein-protein interactions. Anti-estrogens such as tamoxifen inhibit the growth of ER-positive breast cancers by reducing the expression of estrogen-regulated genes. However, anti-estrogen-resistant growth of ER-positive tumors remains a significant clinical problem. Here we show that phosphatidylinositol (PI) 3-kinase and AKT activate ERα in the absence of estrogen. Although PI 3-kinase increased the activity of both estrogen-independent activation function 1 (AF-1) and estrogen-dependent activation function 2 (AF-2) of ERα, AKT increased the activity of only AF-1. PTEN and a catalytically inactive AKT decreased PI 3-kinase-induced AF-1 activity, suggesting that PI 3-kinase utilizes AKT-dependent and AKT-independent pathways in activating ERα. The consensus AKT phosphorylation site Ser-167 of ERα is required for phosphorylation and activation by AKT. In addition, LY294002, a specific inhibitor of the PI 3-kinase/AKT pathway, reduced phosphorylation of ERα *in vivo*. Moreover, AKT overexpression led to up-regulation of estrogen-regulated pS2 gene, Bcl-2, and macrophage inhibitory cytokine 1. We demonstrate that AKT protects breast cancer cells from tamoxifen-induced apoptosis. Taken together, these results define a molecular link between activation of the PI 3-kinase/AKT survival pathways, hormone-independent activation of ERα, and inhibition of tamoxifen-induced apoptotic regression.

Estrogen-induced proliferation of mammary and uterine epithelial cells is primarily mediated by estrogen receptors (ERs),[1] which belong to steroid/thyroid hormone superfamily of transcription factors (1). ERs, through their estrogen-independent and estrogen-dependent activation domains (AF-1 and AF-2, respectively), regulate transcription by recruiting coactivator proteins and interacting with the general transcriptional machinery (2). Tamoxifen, which functions as a cell type-specific anti-estrogen, competitively binds to ERα and inhibits estrogen-stimulated growth of mammary epithelial cells. Depending on the concentration of tamoxifen, growth-arrested cells undergo apoptosis within 24 h or after 72 h of tamoxifen treatment (3). Although most ERα-positive breast cancers initially respond to tamoxifen therapy, tamoxifen-resistant tumors eventually develop (4). It has been shown previously that growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and heregulin confer estrogen-independent growth properties to ERα-positive breast cancer cells (5–7). It is suggested that EGF- and IGF-1-induced mitogen-activated protein kinase (MAPK) phosphorylates Ser-118 of ERα, increases the activity of AF-1, and confers hormone-independent growth (6). However, a recent study indicates that, although prolonged activation of MAPK is growth inhibitory in breast cancer cells, parallel activation of the PI 3-kinase/AKT pathway by EGF and IGF-1 is sufficient to overcome such inhibition (8). Therefore, these growth factors may utilize the PI 3-kinase/AKT pathway to activate ERα and confer hormone-independent growth.

Growth factor-dependent survival of a wide variety of cultured cells types ranging from fibroblasts to neurons is dependent on PI 3-kinase pathway (9). Growth factor-induced activation of transmembrane receptors (IGF-1, EGF, platelet-derived growth factor, basic fibroblast growth factor, and heregulin) results in recruitment of PI 3-kinase to the plasma membrane (9). In the plasma membrane, PI 3-kinase promotes generation of 3′-phosphorylated phosphoinositides, which in turn bind to AKT. AKT bound to phosphoinositides is translocated from cytoplasm to the plasma membrane, where it is activated through phosphorylation. Apart from growth factors, estrogen also activates AKT by triggering the binding of ERα to the p85 regulatory subunit of PI 3-kinase (10). However, induction of AKT by estrogen is cell type-specific, as it is not observed in MCF-7 breast cancer cells (11). Activated AKT promotes cell survival by phosphorylating and modulating the activity of various transcription factors in the nucleus. The tumor suppressor PTEN gene, which dephosphorylates 3′-phosphoryl-

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; 4-HT, 4-hydroxytamoxifen; IGF, insulin-like growth factor; MIC-1, macrophage inhibitory cytokine-1; AF, activation function; EGF, epidermal growth factor; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PR, phenol red; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; TK, thymidine kinase; RARE, retinoic acid response element; DBD, DNA binding domain; CCS, charcoal/dextran-treated serum; AB, amino-terminal A/B region.

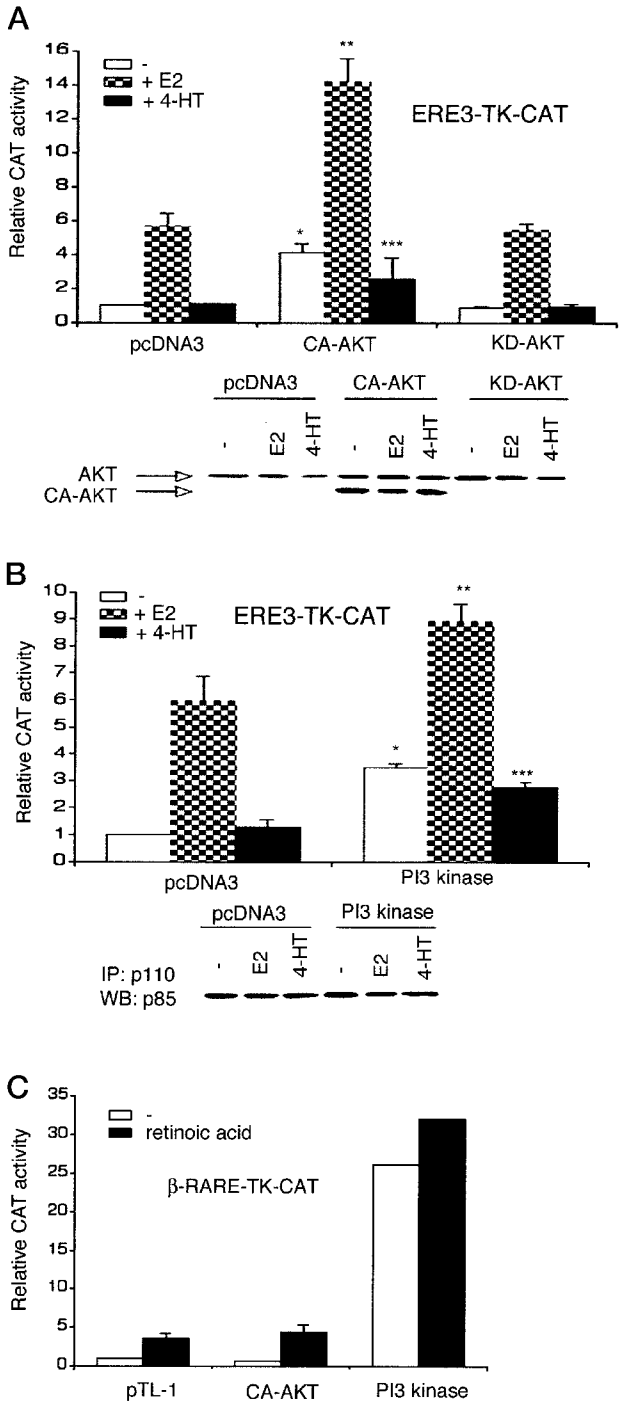

FIG. 1. **AKT specifically increases estrogen-independent activity of ERα.** *A*, MCF-7 cells were transfected with 5 μg of ERE3-TK-CAT and 1 μg of pcDNA3, CA-AKT, or KD-AKT. Cotransfection of AKT led to increases in estrogen-independent activity and further increases in estrogen-stimulated (*E2*, 10^{-8} M) activity of ERα. *, $p = 0.0019$; **, $p = <0.0001$; and ***, $p = 0.07$ for CA-AKT *versus* pcDNA3 in the absence and presence of estrogen and 4-HT, respectively. Western blot analysis of extracts used in a representative CAT assay for CA-AKT expression is shown. *B*, PI 3-kinase-dependent increases in ERE3-TK-CAT activity. Western blot of p85 subunit of PI 3-kinase that coprecipitates with p110 subunit is also shown. *, $p = 0.0019$ for untreated PI 3-kinase *versus* untreated pcDNA3 cells. **, $p = 0.0008$ for estrogen-treated PI 3-kinase *versus* estrogen-treated pcDNA3 cells. ***, $p = 0.0238$ for 4-HT-treated PI 3-kinase cells *versus* 4-HT-treated pcDNA3 cells. C, AKT had no effect on retinoic acid (10^{-6} M) inducible-expression of β-RARE-TK-CAT. In contrast, PI 3-kinase increased β-RARE-TK-CAT activity in the absence of retinoic acid.

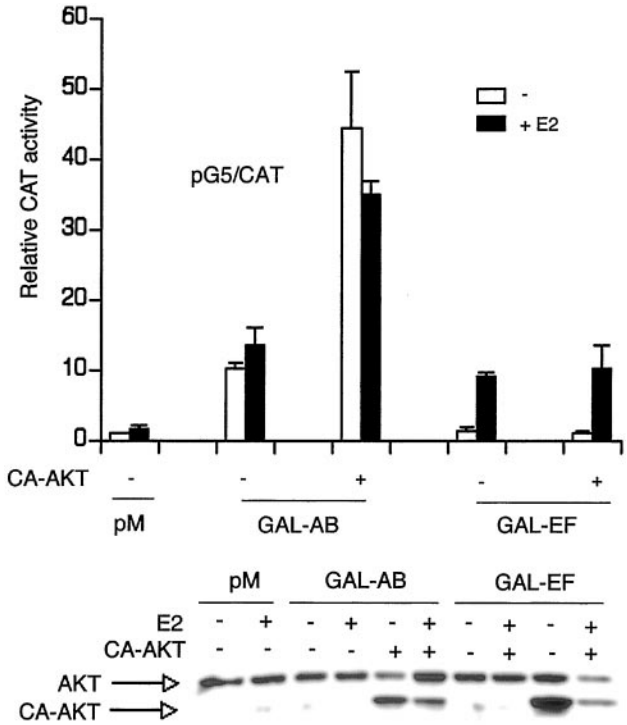

FIG. 2. **AF-1 is target for AKT.** COS-1 cells were transfected with GAL-DBD (pM), GAL-AB, or GAL-EF along with pG5/CAT and RSV/β-galactosidase. GAL-AB activity in the absence of CA-AKT is set arbitrarily as 10 units. GAL-AB activity increased by 3.5-fold in the presence of CA-AKT. GAL-EF activity was not affected by AKT and was appreciated only in estrogen-treated (10^{-8} M) cells. CA-AKT expression level in transfected cells is shown.

ated phosphoinositides *in vivo*, inhibits AKT activation (12). Several clinical and laboratory observations prompted us to study whether ERα is the target of AKT and whether AKT protects breast cancer cells against tamoxifen-induced apoptosis. For example, PI 3-kinase and AKT amplification is observed in breast and ovarian cancer (13–15). Cowden disease patients, who have a germ line-inactivating PTEN mutation, display increased breast cancer risks (16). Similarly, PTEN mutation is observed in endometrial cancer (17). Moreover, IGF-1 and heregulin, whose overexpression correlates with tamoxifen resistance, activate AKT (5, 7). Finally, serine at amino acid position 167 of ERα is a consensus AKT phosphorylation site (R*X*R*XX*(S/T)) (9). In this report, we show that AKT phosphorylates Ser-167 of ERα and confers tamoxifen resistance.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells were grown in minimal essential medium (MEM) containing phenol red (PR), 10% fetal calf serum (FCS), streptomycin, and penicillin. COS-1 cells were maintained in Dulbecco's modified MEM (DMEM) including PR, 10% FCS, streptomycin, and penicillin.

Recombinant Plasmids, DNA Transfections, and CAT Assays—MCF-7 and COS-1 cells were passed 24 h prior to transfection in PR-free DMEM + 5% charcoal/dextran-treated serum (CCS) and transfected via the calcium phosphate method. The β-galactosidase expression vector RSV/β-galactosidase (2 μg) was cotransfected as an internal control. In all transfections, the total amount of expression vector was kept constant by substituting with appropriate empty expression vector. Fresh media (PR-free MEM + 5% CCS for MCF-7, PR-free DMEM + 5% CCS for COS-1 cells) along with appropriate drugs were added 24 h after transfection. Cells were harvested 36 h after transfection and CAT activity in equal numbers of β-galactosidase units was determined as described previously (18). ERE3-TK-CAT, GAL-AB and GAL-EF were gifts from P. Chambon and described previously (19). The CA-AKT expression vector that lacks pleckstrin homology domain (amino acids 4–129) of AKT and KD-AKT expression vector that contains S179M

substitution have been described previously (20) and were gifts from R. Roth. COS-1 cell transfections were performed with the original SV40 enhancer-promoter-driven CA-AKT and KD-AKT expression vectors. pcDNA3 derivatives of CA-AKT and KD-AKT were generated and used in MCF-7 cell transfections. Constitutively active PI 3-kinase vector, which contains murine p110α cDNA with avian Src myristoylation signal at the N terminus (21), was a gift from S. Boswell. PTEN expression vector was a gift from J. Dixon.

Phosphorylation of GST-AB by AKT in Vitro—Amino acids 1–184 of ERα were cloned in frame to pGEX-2T vector to obtain recombinant GST-AB. AKT-kinase assay with recombinant GST, GST-AB, or AB obtained after cleaving GST-AB with thrombin in the presence of [γ-^{32}P]ATP was performed as per AKT manufacturer's recommendations (Upstate Biotechnology, Inc.).

AKT-overexpressing Clones and Growth Analysis—MCF-7 cells were transfected with either empty pcDNA3 or pcDNA3 vector encoding CA-AKT. Transfected cell populations were selected using G418 (0.6 mg/ml) and CA-AKT expression in individual G418-resistant colonies was verified by Western analysis. MCF-7 control (pcDNA-1) and CA-AKT (CA-AKT-4) cells were plated in MEM with PR and 10% FCS at a density of 2×10^5 cells/60-mm plate. After 24 h, fresh medium containing either 10^{-8} M estradiol or 10^{-6} M 4-HT or 10^{-7} M ICI-182,780 was added and replaced every 48 h with appropriate drugs. At 72, 96, and 120 h, cells were collected by trypsinization, stained with trypan blue, and counted twice using a hemocytometer. All experiments were performed in triplicate.

Cell Cycle Analysis and Quantitation of Apoptosis—MCF-7 control and CA-AKT cells were plated at a density of 1×10^5 cells/60-mm dish in MEM with PR + 10% FCS. After 24 h, fresh medium was added, along with 10^{-6} M 4-HT. Cell cycle analysis utilizing propidium iodide staining was performed at specific intervals. Apoptosis of untreated and 4-HT-treated (10^{-6} M for 72 h) control and CA-AKT cells was measured by annexin V labeling and fluorescence microscopy, as per manufacturer's recommendations (annexin V-EGFP, BioVision).

Western Blot Analysis and Immunoprecipitation—Western blotting/immunoprecipitation of cell lysates with antibodies against cyclin D1, PTEN (Santa Cruz), Bcl-X_L, BAX, BAK (Trevigen), Bcl-2 (PharMingen), PI3K-p110, p85 (Upstate Biotechnology, Inc.), AKT (New England Biolabs), and α-tubulin (Sigma) was performed as per manufacturer's recommendations. ERα antibodies were from either P. Chambon's laboratory or from Chemicon.

Orthophosphate Labeling and Immunoprecipitation of ERα—ERα cDNA was first cloned into pCMV4 expression vector, which is similar to pcDNA3, except that it contains a translational enhancer from alfalfa mosaic virus 4. 293 cells were transfected with either empty expression vector or pCMV4-ERα. After maintaining cells for 48 h in PR-free DMEM with 5% CCS, cells were serum-starved in PR-free, phosphate-free DMEM for 24 h. Cells were incubated with orthophosphate and either Me_2SO or 20 μM LY294002. IGF-1 (100 ng/ml, R&D Systems) was added after 1 h, and cells were harvested after an additional 1 h. ERα was immunoprecipitated and subjected to autoradiography, followed by Western blotting with ERα antibody.

Statistical Analysis—Data were analyzed with StatView (version 4.1). Analysis of variance was employed to determine p values between mean measurements. A p value < 0.05 was deemed significant. Error bars on all histograms in this text represent standard deviations between measurements from duplicate experiments.

RESULTS

AKT Confers Ligand-independent Activity to ERα—To investigate the ability of AKT to directly regulate ERα activity, ERα-positive MCF-7 breast cancer cells were transiently transfected with a CAT reporter gene under the control of a thymidine kinase promoter and three ERE elements (ERE3-TK-CAT). Estrogen increased ERE3-TK-CAT activity 6-fold, whereas 4-hydroxytamoxifen (4-HT) had no effect (Fig. 1*A*). Cotransfection of a constitutively active AKT expression vector (CA-AKT) resulted in 4.5- and 15-fold increases in ERE3-TK-CAT activity in the absence and presence of estrogen, respectively (Fig. 1*A*). Cotransfection of a catalytically inactive AKT vector (KD-AKT) affected neither estrogen-dependent nor estrogen-independent ERE3-TK-CAT activity. These results suggest that AKT confers estrogen-independent activity and further increases estrogen-stimulated activity of ERα. Note that

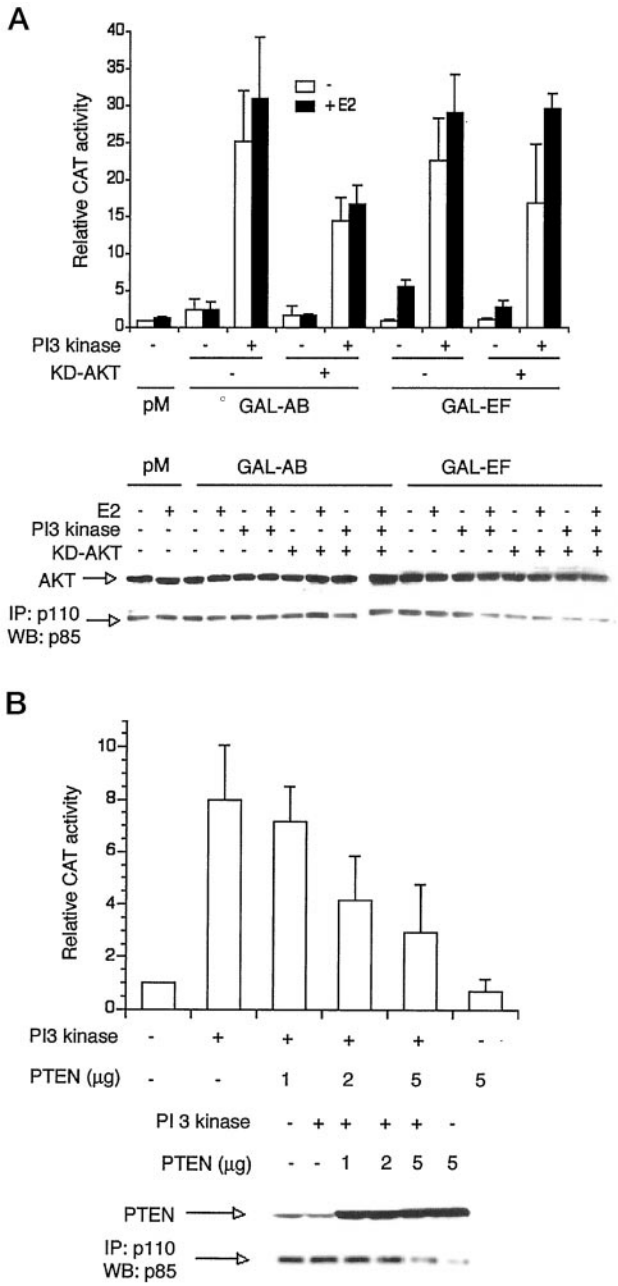

FIG. 3. **PI 3-kinase activates AF-1 and AF-2 via AKT-dependent and AKT-independent mechanisms, respectively.** COS-1 cells were transfected with the indicated plasmids and pG5/CAT reporter. *A*, note that PI 3-kinase increased both GAL-AB and GAL-EF activity. In addition, KD-AKT reduced PI 3-kinase-induced GAL-AB activity, indicating that PI 3-kinase utilizes AKT to activate AF-1. *B*, PTEN reduced PI 3-kinase-induced GAL-AB activity, suggesting that activation of AF-1 by PI 3-kinase requires the presence of AKT. Expression levels of endogenous and transfected genes are shown.

AKT-mediated estrogen-independent ERE3-TK-CAT activity was reduced to 2.5-fold by 4-HT.

A substantial (4-fold) increase in estrogen-independent activity was observed when ERE3-TK-CAT was transfected along with PI 3-kinase, the upstream activator of AKT (Fig. 1*B*). PI 3-kinase-induced ERE3-TK-CAT activity was reduced to 2.5-fold by 4-HT. CA-AKT had no effect on basal and retinoic acid-inducible expression of β-RARE-TK-CAT (a TK promoter with a retinoic acid response element from the β-retinoic acid receptor) (18). In contrast, PI 3-kinase increased β-RARE-TK-CAT activity in the absence of retinoic acid (Fig. 1*C*). These results suggest that, although AKT overexpression leads to a

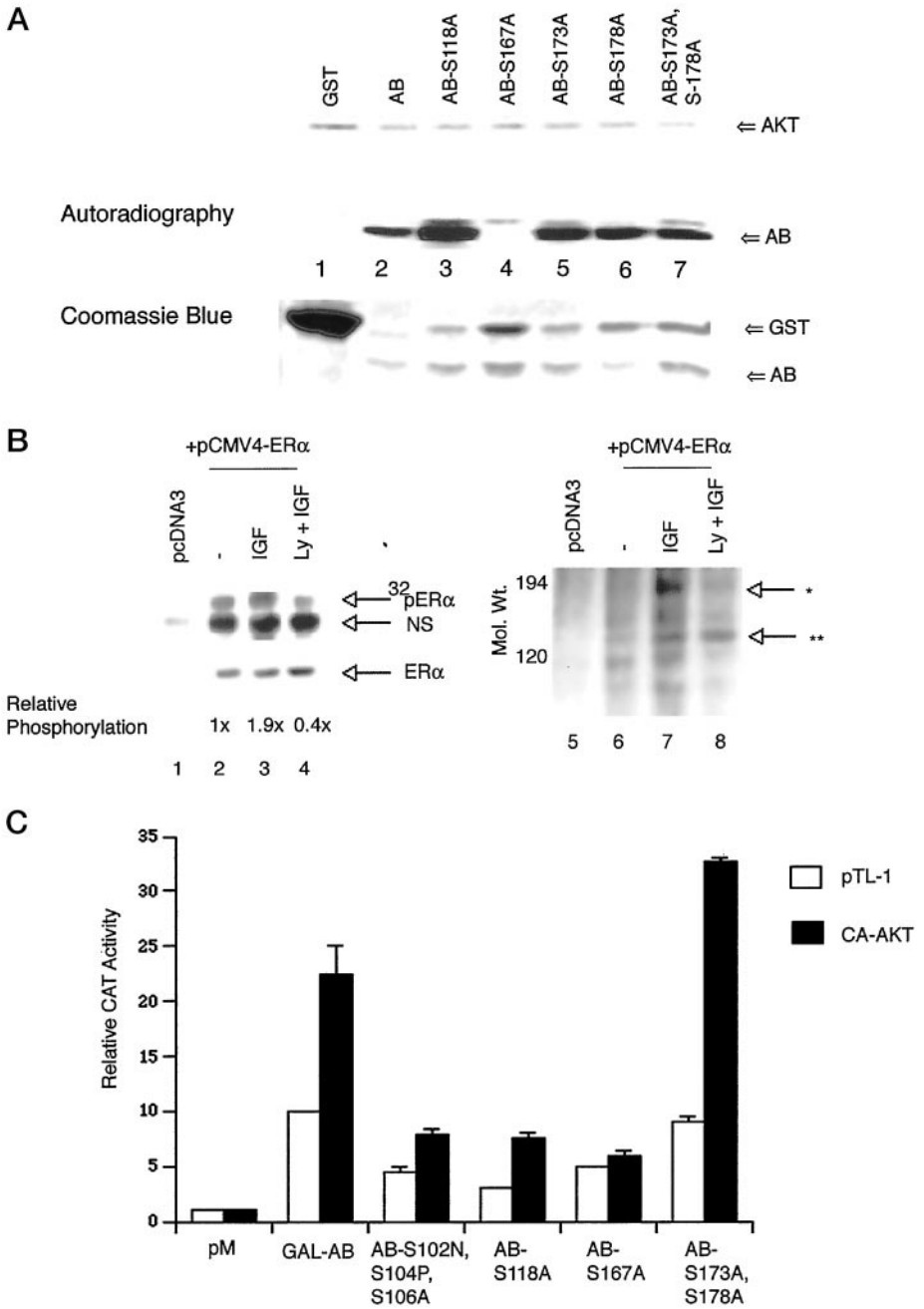

FIG. 4. **AKT directly phosphorylates AF-1.** *A*, phosphorylation of recombinant AB by purified AKT *in vitro*. Recombinant GST, AB, or AB mutants were incubated with recombinant AKT and [γ-^{32}P]ATP. Kinase reactions were run on an SDS-PAGE, transferred to nitrocellulose, and subjected to both autoradiography (*top*) and staining with Coomassie Blue (*bottom*). *B*, effect of LY294002 on ERα phosphorylation. Orthophosphate-labeled ERα from 293 cells transfected with either vector alone or pCMV4-ERα was identified by immunoprecipitation and autoradiography (*lanes 1–4*). Western blot analysis of the same blot for ERα protein is also shown. Relative phosphorylation in each reaction was calculated by densitometric scanning and normalizing for ERα level. Orthophosphate-labeled proteins that coprecipitate with ERα are also shown (*lanes 5–8*). *NS*, nonspecific. *C*, serine 167 in the AB domain is essential for AKT-mediated activation. COS-1 cells were transfected with either GAL-AB or mutants as in Fig. 2. AB-S167A mutant in which serine 167 is mutated to alanine was not activated by CA-AKT. Wild type and mutant GAL-AB proteins are expressed at a similar level in transfected cells (data not shown).

specific increase in ERα activity, PI 3-kinase overexpression may lead to a global change in nuclear receptor activity, possibly through activation of coactivators (2, 22).

AKT Increases AF-1 Activity—To determine the activation domains of ERα required for estrogen-independent activation by AKT, we utilized expression vectors that code for a fusion protein containing the DNA binding domain (DBD) of yeast transcription factor GAL-4 and either the AF-1 domain (GAL-AB) or AF-2 domain of ERα (GAL-EF) (19). A CAT reporter gene under the control of a TATA box and five binding sites for GAL-4 DBD (pG5/CAT) was cotransfected with either GAL(DBD) alone (pM), GAL-AB, or GAL-EF with or without CA-AKT. COS-1 cells were used because they provide an ERα-free background. CA-AKT increased GAL-AB activity by ~3.5-fold, which was not influenced by estrogen (Fig. 2). GAL-EF activity was observed only in estrogen-treated cells and was not affected by AKT. These results demonstrate that AKT specifically increases AF-1 activity and are consistent with previously described EGF- and IGF-1-regulated enhancement of AF-1 activity (6).

PI 3-Kinase Increases Both AF-1 and AF-2 Activity—PI 3-kinase, apart from AKT, activates other signaling pathways including Jun N-terminal kinase, protein kinase Cλ and protein kinase Cζ, which may contribute to a PI 3-kinase-mediated increase in ERα activity (23–25). To test this hypothesis, we transfected COS-1 cells with pG5/CAT, GAL-AB, or GAL-EF and a constitutively active PI 3-kinase (Fig. 3*A*). PI 3-kinase increased GAL-AB activity by ~8-fold. Cotransfection with KD-AKT resulted in reduction of PI 3-kinase-induced GAL-AB activity. PTEN, a negative regulator of PI 3-kinase-mediated activation of AKT (12), also showed a concentration-dependent reduction of PI 3-kinase-induced GAL-AB activity (Fig. 3*B*). For unknown reasons, we observed reduced levels of PI 3-kinase p110·p85 complex in cells transfected with 5 μg of PTEN (Fig. 3*B*, *bottom panel*). Therefore, it is not clear whether reduction in GAL-AB activity by PTEN involves only its lipid

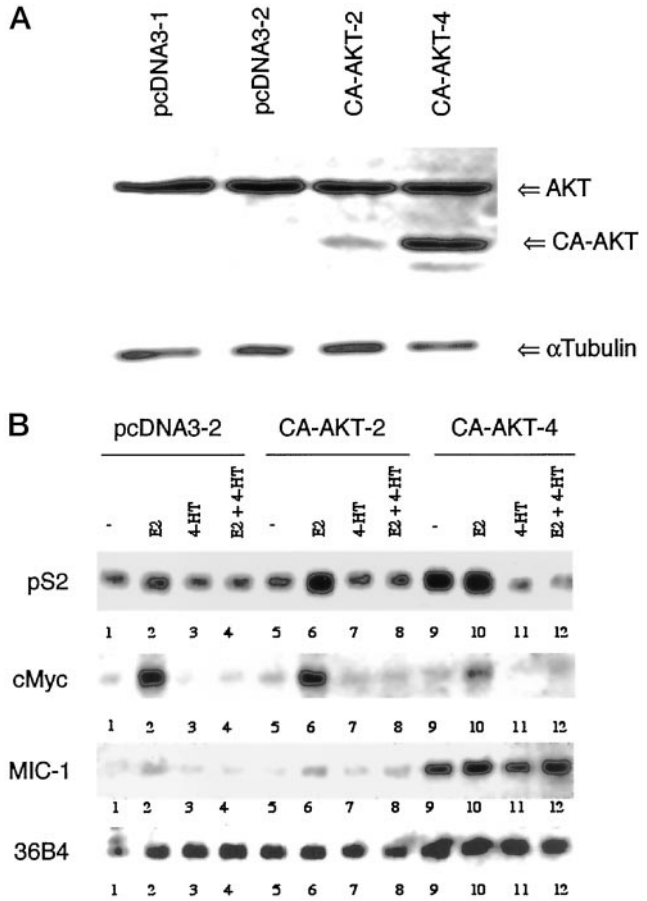

FIG. 5. **AKT increases pS2 and MIC-1 expression.** *A*, MCF-7 cells stably overexpressing AKT (CA-AKT-2, CA-AKT-4) and empty vector (pcDNA3–1, pcDNA3–2) were developed and analyzed for CA-AKT expression by Western blotting. Endogenous AKT and transfected constitutively active AKT (CA-AKT) are indicated. *B*, AKT increases pS2 but not c-Myc expression. Total RNA (20 μg) from cells grown in PR-free MEM + CCS with or without treatment for 1 h was subjected to Northern analysis with indicated probes. The integrity of RNA was verified by reprobing the blot with 36B4 ribosomal protein gene cDNA.

phosphatase activity or other biological activity. Nonetheless, PTEN overexpression resulted in reduction in AKT activity in transfected cells (data not shown). Interestingly, GAL-EF, which was not induced by AKT (Fig. 2), was activated by PI 3-kinase both in the absence and presence of estrogen (Fig. 3*A*). Moreover, KD-AKT failed to reduce GAL-EF activation by PI 3-kinase, suggesting that activation of AF-1 (but not AF-2) by PI 3-kinase is, at least in part, AKT-dependent (Fig. 3*A*).

Ser-167 of ERα Is Essential for Phosphorylation and Activation by AKT—Phosphorylation of the AF-1 domain of ERα by AKT was investigated using purified AKT and a recombinant protein containing the AF-1 domain fused to glutathione *S*-transferase (GST-AB). GST and GST-AB proteins were incubated with [γ-^{32}P]ATP and recombinant AKT. GST-AB but not GST was phosphorylated by AKT, thereby confirming direct phosphorylation of AF-1 by AKT (data not shown). Mass spectrometry followed by mutation analysis revealed that AKT fortuitously phosphorylates Ser-173 or Ser-178 of AF-1 within the context of GST-AB fusion protein (data not shown). Therefore, purified wild type AB or mutants lacking GST were used in *in vitro* kinase assays. Mutation of Ser-167 (AB-S167A) but not Ser-118 (AB-S118A), Ser-173 (AB-S173A), Ser-178 (AB-A178A), and Ser-173/Ser-178 (AB-S173A,S178A) to alanine prevented phosphorylation by AKT (Fig. 4*A*). Ser-167 is a consensus AKT phosphorylation site (R*X*R*XX*(S/T)), which is also phosphorylated by p90^{RSK1} (26), and is the major phosphorylation site of ERα in MCF-7 cells (27).

We next determined whether basal or IGF-1-induced phosphorylation of ERα is dependent on PI 3-kinase/AKT pathway. Human embryonic kidney cells 293 transfected with ERα expression vector were labeled with [^{32}P]orthophosphate and treated with Me_2SO, IGF-1, or LY294002 + IGF-1. LY294002 is a specific inhibitor of PI 3-kinase/AKT pathway. Although IGF-1 treatment led to only marginal increase in overall ERα phosphorylation, LY294002 inhibited ERα phosphorylation (Fig. 4*B*). The effect of LY294002 is specific to ERα, as phosphorylation of an unknown protein that coprecipitates with ERα was similar in both untreated and LY294002-treated cells (Fig. 4*B*, *lanes 5–8*, indicated by **). These results further suggest the involvement of PI 3-kinase/AKT pathway in phosphorylation of ERα.

We next compared AKT-mediated activation of wild type GAL-AB and GAL-AB mutants lacking Ser-102, Ser-104, and Ser-106 (AB-S102N,S104P,S106A), Ser-118 (AB-S118A), Ser-167 (AB-S167A), or Ser-173 and Ser-178 (AB-S173A,S178A). Only AB-S167A did not respond to AKT, suggesting that Ser-167 is essential for activation of AF-1 by AKT (Fig. 4*C*). Mutation of Ser-167 to alanine also reduced estrogen-independent and/or estrogen-dependent activity of full-length ERα in COS-1, MDA-MB-231, and HeLa cells (data not shown). AKT-mediated activation of AB-S102N, S104P, S106A, and AB-S118A was also lower than the wild type, which could be due to reduced basal activity of these mutants. Alternatively, activation of AF-1 by AKT is partially dependent on phosphorylation of AB by cdk2/cyclinA or MAPK (6, 28). In this regard, we have observed that mutation of both cdk2/cyclin A and AKT phosphorylation sites severely compromised ligand-independent activity of full-length ERα in MDA-MB-231 cells.[2]

Endogenous Estrogen-regulated pS2 Gene Expression Is Up-regulated by AKT—To study whether CA-AKT induces the expression of endogenous estrogen-regulated genes, we generated MCF-7 cells overexpressing CA-AKT (Fig. 5*A*). We measured the expression of pS2 and c-Myc in untreated, estrogen-, 4-HT-, and estrogen+4-HT-treated cells. The basal level of pS2 was higher in CA-AKT-4 cells compared with pcDNA3–2 and CA-AKT-2 cells (Fig. 5*B*). Estrogen-inducible expression of pS2 was higher in both CA-AKT-2 and CA-AKT-4 cells compared with pcDNA3 cells. Note that CA-AKT-4 cells express higher level of AKT than CA-AKT-2 cells (Fig. 5*A*). As with the results of transient transfection assays, constitutive and estrogen-inducible expression of pS2 was repressed by 4-HT. Interestingly, AKT overexpression did not result in changes in c-Myc expression.

Screening of Atlas Human Cancer cDNA expression array using RNA from pcDNA3 and CA-AKT cells revealed up-regulation of macrophage inhibitory cytokine (MIC-1) by AKT (29). This was confirmed by Northern blotting (Fig. 5*B*). MIC-1 expression was observed only in CA-AKT-4 cells, which was increased modestly by estrogen. Interestingly, 4-HT did not suppress MIC-1 expression. Taken together, these results suggest that AKT increases either constitutive or estrogen-inducible expression of specific estrogen-responsive genes.

AKT Confers Tamoxifen Resistance to MCF-7 Cells—Because AKT increases AF-1 activity and AF-1 is implicated in tamoxifen resistance, we studied the 4-HT sensitivity of pcDNA3–1 and CA-AKT-4 cells (Fig. 6*A*). Growth rates for untreated, estrogen- (10^{-8} M), 4-HT- (10^{-6} M), and estrogen+4-HT-treated cells were determined at specific intervals. As expected, estrogen stimulated the growth of both pcDNA3 and CA-AKT cells.

[2] H. Nakshatri, manuscript in preparation.

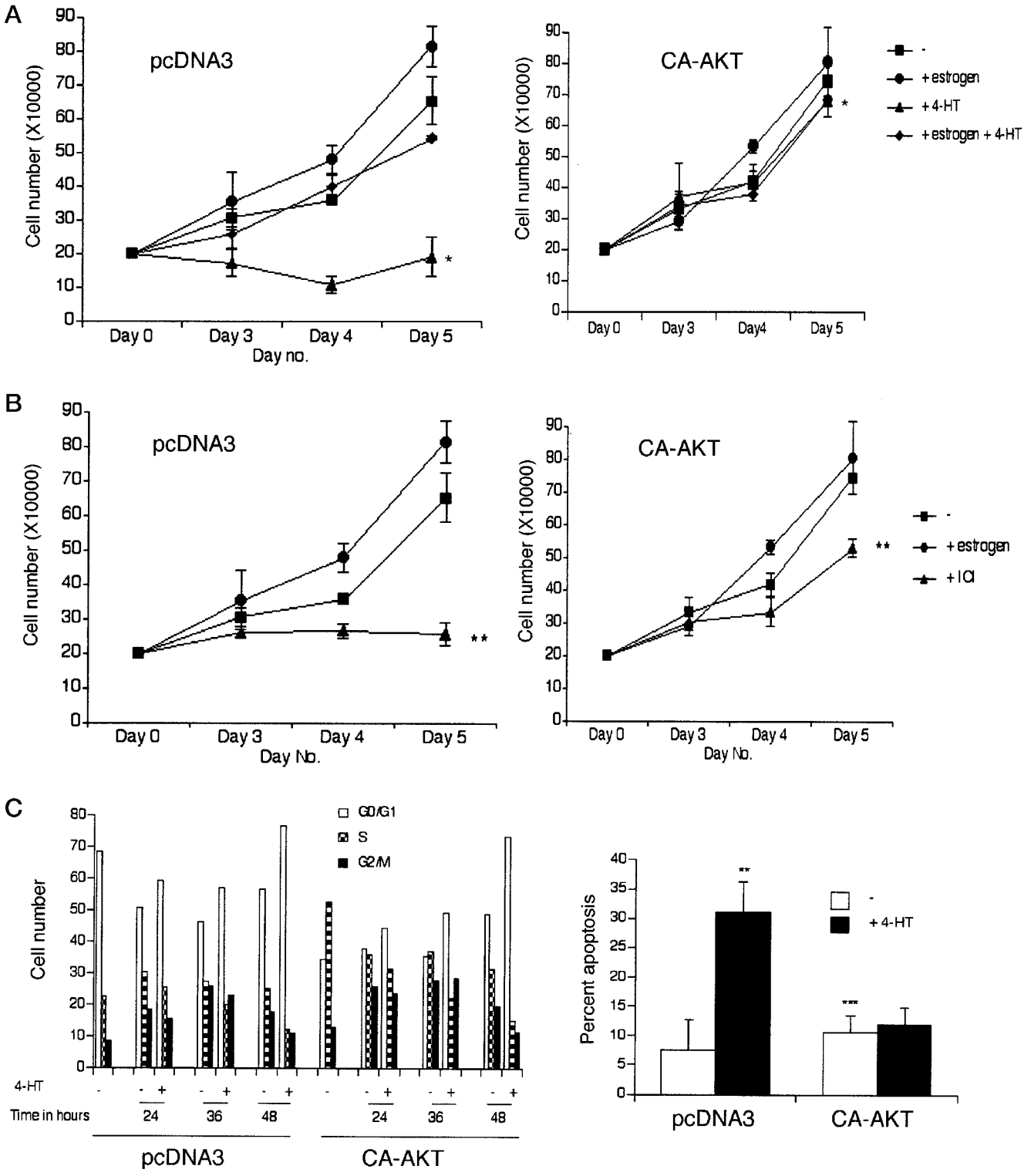

FIG. 6. **AKT overexpression confers tamoxifen resistance through inhibition of tamoxifen-induced apoptosis.** *A*, growth rates of untreated and estrogen-treated (10^{-8} M) and/or 4-HT-treated (10^{-6} M) pcDNA3–1 and CA-AKT-4 cells were measured by trypan blue exclusion and manual counting at specific intervals. CA-AKT cells were significantly more resistant to 4-HT than pcDNA3 cells. *, $p < 0.0004$ for 4-HT-treated pcDNA3 *versus* 4-HT-treated CA-AKT cells (day 5). *B*, growth rates of untreated and estrogen-treated (10^{-8} M) or ICI-182,780-treated (10^{-7} M) pcDNA3–1 and CA-AKT-4 cells. **, $p = 0.0001$ for untreated *versus* ICI-treated pcDNA3 cells. **, $p = 0.0078$ for untreated *versus* ICI-treated CA-AKT-4 cells. *C*, pcDNA3 and CA-AKT cells arrest at G_0/G_1 phase at a similar rate after 4-HT treatment. Cell cycle distribution was determined by flow cytometric analysis of propidium iodide-stained cells. *D*, AKT inhibits 4-HT-induced apoptosis. Cells were treated with 4-HT (10^{-6} M) for 72 h, and rate of apoptosis was measured by annexin V labeling. **, $p < 0.0001$ for percentage of apoptosis in 4-HT-treated CA-AKT *versus* 4-HT-treated pcDNA3 cells. ***, $p = 0.59$ for untreated *versus* 4-HT-treated CA-AKT cells

Also note that we reproducibly observed ~20% increase in the basal growth rate of untreated CA-AKT compared with untreated pcDNA3 cells. Significantly, 4-HT was more efficient in inhibiting the growth of pcDNA3 compared with CA-AKT cells, indicating that AKT reduces 4-HT sensitivity of MCF-7 cells. In contrast to 4-HT, CA-AKT cells were significantly growth-in-

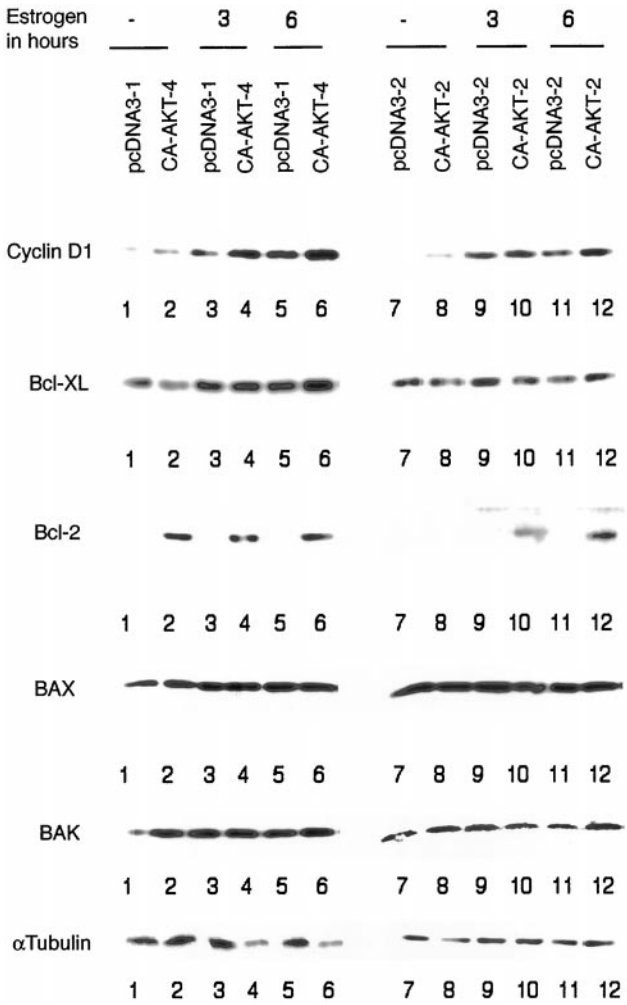

FIG. 7. **AKT up-regulates Bcl-2.** Whole cell extracts from untreated and estrogen-treated (10^{-8} M) pcDNA3 and CA-AKT cells were subjected to Western analysis using the indicated antibodies. AKT overexpression led to specific up-regulation of the anti-apoptotic Bcl-2 gene.

hibited by the pure anti-estrogen ICI-182,780 (Fig. 6*B*), which inhibits ERα activity by preventing DNA binding and enhancing degradation (30). These results suggest that modulation of ERα activity by AKT is responsible for 4-HT resistance of CA-AKT cells, although we can not completely rule out the involvement of ERα-independent anti-apoptotic activity of AKT in 4-HT resistance.

Reduced sensitivity of CA-AKT cells to 4-HT could be due to either loss of 4-HT-induced cell cycle arrest at G_0/G_1 or inhibition of 4-HT-induced apoptosis. To resolve this issue, we measured the cell cycle distribution of untreated and 4-HT-treated pcDNA3 and CA-AKT cells. Both pcDNA3 and CA-AKT cells accumulated at G_0/G_1 phase at a similar rate after 4-HT treatment (Fig. 6*C*). Apoptosis of untreated and 4-HT-treated (10^{-6} M for 72 h) pcDNA3 and CA-AKT cells were measured by annexin V labeling and fluorescence microscopy (Fig. 6*D*). A significantly higher percentage of pcDNA3 cells (31%) were apoptotic when compared with CA-AKT cells (12%) after 4-HT treatment. AKT therefore inhibits 4-HT-induced apoptosis but not cell cycle arrest of MCF-7 cells.

AKT Increases Bcl-2 but Not Cyclin D1 Expression—Western blot analysis of untreated and estrogen-treated cell extracts was performed to identify estrogen-regulated cell cycle and anti-apoptotic genes that are overexpressed in CA-AKT cells (Fig. 7). Cyclin D1 expression proved estrogen-inducible in both cell types. AKT had no significant effect on cyclin D1 expression. The known anti-apoptotic and estrogen-inducible Bcl-2 gene (31) was expressed at very low levels and did not appear to be up-regulated by estrogen in pcDNA3 cells. In CA-AKT-2 cells, which expresses lower levels of AKT than CA-AKT-4 cells, estrogen dramatically increased Bcl-2 expression. In CA-AKT-4 cells, Bcl-2 is constitutively overexpressed. Expression of anti-apoptotic Bcl-X_L and pro-apoptotic BAK and BAX was similar in both pcDNA3 and CA-AKT cells. These results suggest that AKT specifically increases Bcl-2 expression in MCF-7 cells. Because Bcl-2 overexpression in CA-AKT-2 cells was observed only in the presence of estrogen, it is likely that increased Bcl-2 expression in CA-AKT cells requires ERα activity.

DISCUSSION

In this report, we demonstrate that PI 3-kinase/AKT signaling pathway modulates ERα activity *in vivo*, which correlates with phosphorylation of Ser-167 by AKT *in vitro*. Recent studies indicate that estrogen promotes association of ERα with IGF-1 receptor and p85 subunit of PI 3-kinase in the plasma membrane, which leads to AKT activation (10, 32). Our results suggest that AKT serves as a functional link between membrane-associated and nuclear ERα. By phosphorylating Ser-167, AKT may modulate coactivator:AF-1 and/or corepressor:AF-1 interactions in the nucleus. Consistent with this possibility, previous studies have shown that phosphorylation of Ser-118 of ERα alters both coactivator:AF-1 and corepressor:AF-1 interactions (33, 34).

AF-1 region of ERα contains phosphorylation sites for a number of kinases including MAPK and cyclin A/cdk2 (6, 28, 35). Some of these sites are conserved between ERα and ERβ (36). However, AKT phosphorylation site is present only in ERα (36). ERs exist as homodimers as well as ERα·ERβ heterodimers, and these combinations have different affinity for EREs (37, 38). Therefore, induction of ERE containing genes upon activation of AKT may be cell type-dependent. Furthermore, regulation of ERα activity by AKT may be controlled by $p90^{RSK1}$ as it can also phosphorylate Ser-167 (26). Finally, MAPK may indirectly control AKT-mediated activation of ERα as it can activate $p90^{RSK1}$ (39).

We observed up-regulation of pS2, MIC-1, and Bcl-2 but not c-Myc in CA-AKT-overexpressing cells (Fig. 5*B*). These results suggest that the ability of AKT to induce ERE-containing genes is promoter context-dependent. Because 4-HT repressed pS2 but not MIC-1 and Bcl-2 expression in CA-AKT cells, promoter context may also play a role in determining the ability of 4-HT to suppress AKT-mediated activation of estrogen-responsive genes (Fig. 5*B* and data not shown). Both pS2 and Bcl-2 are well characterized estrogen-regulated genes, whereas MIC-1 promoter is yet to be characterized. While induction of pS2 by estrogen requires an ERE (40), activation of Bcl-2 by estrogen requires a SP-1 binding site, cAMP response element-binding protein binding site, and an ERE in the Bcl-2 promoter (31, 41). Interestingly, cAMP response element-binding protein binding site is required for AKT-mediated increase in Bcl-2 (42). Therefore, it is likely that the ability of 4-HT to repress AKT-mediated activation of ERE-containing promoters is determined by promoter elements other than ERE. Alternatively, AKT may activate certain ERE-containing promoters independent of ERα, which is not subject to repression by 4-HT. Additional experiments with dominant negative mutants of ERα (43, 44) are essential to distinguish between these possibilities.

Our results indicate that activation of the PI 3-kinase/AKT pathway leads to increased Bcl-2 expression, which correlates with 4-HT resistance in breast cancer cells. Consistent with our results, Her2/Neu-mediated 4-HT resistance in MCF-7 correlates with Bcl-2 overexpression (45). However, the results of

our cell culture studies are inconsistent with clinical observations that correlate high Bcl-2 expression with favorable response to tamoxifen therapy and prolonged disease-free intervals (46). It is possible that Bcl-2 works in concert with additional anti-apoptotic proteins to confer tamoxifen resistance, which is in accordance with the proposal that overall level of Bcl-2 family anti-apoptotic proteins dictates cellular response to therapy (47).

The PI 3-kinase/AKT pathway is the major survival pathway for a wide variety of cultured cell types (9). ERα may be the central element in this survival pathway, at least in certain cell types. Broader implications of our results are on endometrial cancers as well as on Cowden's disease patients, who show a 30–50% incidence of breast cancer in affected females (16). In both neoplastic conditions, AKT activity is increased due to mutation of PTEN. The PI 3-kinase/AKT pathway, therefore, may be an ideal target for therapeutic intervention in these cancers.

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Phosphatidylinositol 3-Kinase/AKT-mediated Activation of Estrogen Receptor α: A NEW MODEL FOR ANTI-ESTROGEN RESISTANCE

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