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Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ERα) via Interaction between ERα and PI3K[1]

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Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ERα) positive, which prompted us to examine whether AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo*, but it does not phosphorylate a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen resistance. Moreover, we found that ERα binds to the p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ERα and PI3K/AKT2 pathway (ERα-PI3K/AKT2-ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ERα[3] induces a cascade of events, including the release of accessory proteins (*e.g.*, the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor-α that are known to stimulate mammary cell proliferation (1). ERα is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ERα has a NH_2-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ERα is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ERα in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ERα, so as to inhibit ERα-mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ERα. Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ERα-positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ERα activity through activation of protein kinases resulting in phosphorylation of ERα (7).

It has been well documented that phosphorylation of ERα is essential for the activation of ERα after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ERα is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ERα, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH_2 terminus within the AF-1 region. Phosphorylation of ERα at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ERα (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and $pp90^{rsk1}$, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ERα on tyrosine 537, which is required for ERα dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ERα at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ERα plays a crucial role in transducing cellular signals. It has been convincingly shown that ERα activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K^+ and Ca^{2+} channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ERα has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

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[3] The abbreviations used are: ERα, estrogen receptor α; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione *S*-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKKα, Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ERα at Ser-167 and activates ERα-mediated transcription in a PI3K-dependent manner. ERα binds to the p85α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at −70°C. ERα-negative epithelial HEK293 and COS7 cells and ERα-positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C. The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-10 mM $MnCl_2$-1 mM DTT, all containing 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of L-a-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; BIOMOL) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110α and p85α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ERα-S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ERα and GST-ERα-S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ERα fusion proteins were purified as described previously (21).

***In Vivo* [^{32}P]P_i Cell Labeling.** Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P_i (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ERα was immunoprecipitated with monoclonal anti-ERα or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ERα was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ERα, as well as pCMV-βgal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β-galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β-galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1*A*). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

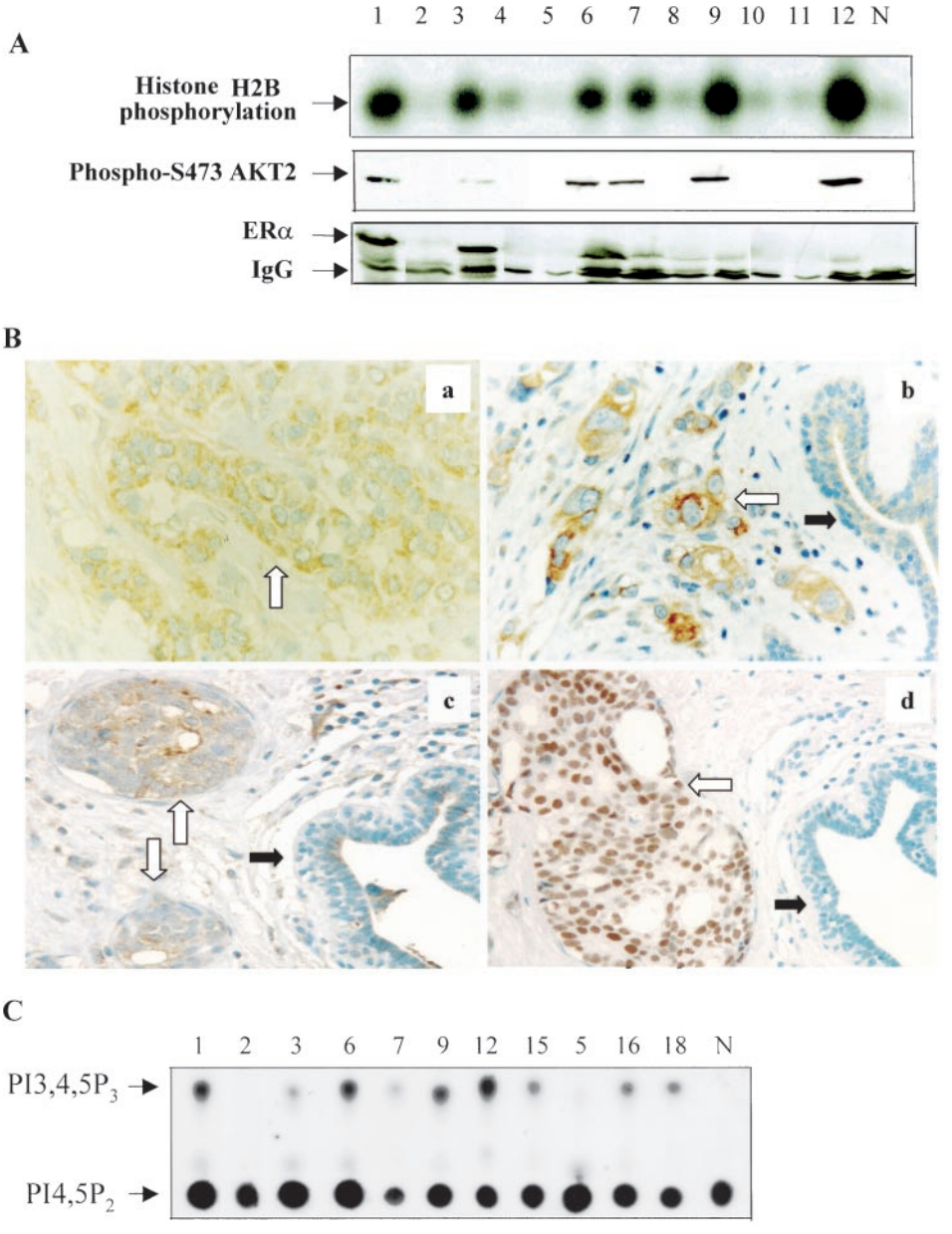

Fig. 1. Activation of AKT2 in human primary breast cancers. *A* (*top panel*), *in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ERα immunoprecipitates with anti-phospho-Ser473 Akt and anti-ERα antibodies, respectively. *B*, immunochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a–c*) and anti-ERα (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (*white arrows*), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (*black arrows*). Photomicrographs *c* and *d* are the same specimen but different sections. *C*, *In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P_2 to PI-3,4,5-P_3 was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26 specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ERα antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ERα positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ERα, suggesting that activated PI3K/AKT2 could be involved in the regulation of ERα activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ERα-mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ERα. Moreover, several other signal molecules, including protein kinase A, casein kinase II, $pp90^{rsk1}$, and MEKK1/p38, have been

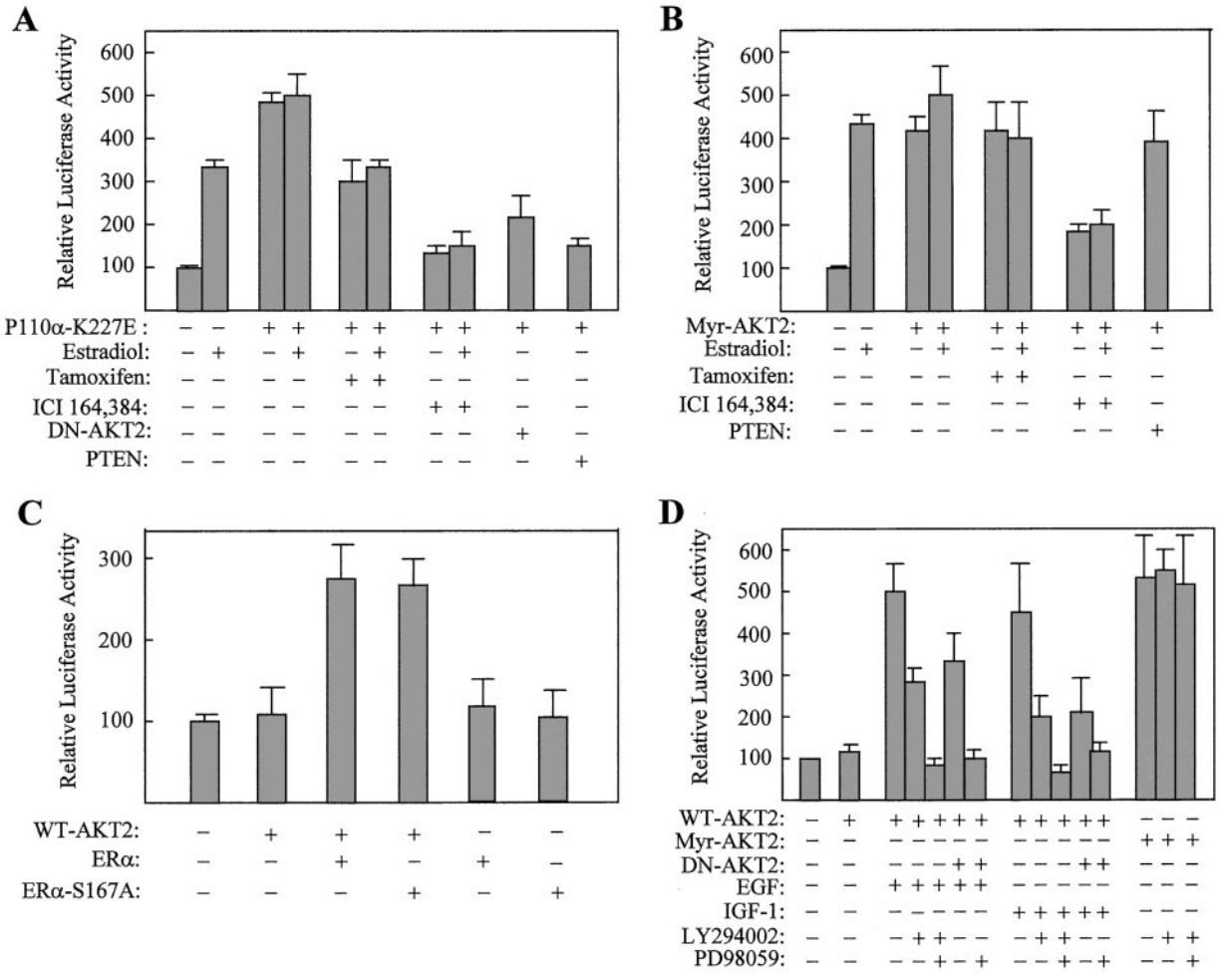

Fig. 2. AKT2 and PI3K activate ERα transcriptional activity. *A–D*, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β-galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β-galactosidase activity.

shown to activate ERα-mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ERα positive, we investigated whether AKT2 and PI3K regulate ERα-mediated transcription. ERα-positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β-galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110α (p110α-K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110α-K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110α-induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2*A*). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110α-K227E and myr-AKT2-stimulated ERα activity (Fig. 2, *A* and *B*), suggesting that PI3K/AKT2-increased ERα transcriptional activity is regulated by phosphorylation of ERα within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ERα (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that negatively regulates PI3K, inhibited constitutively active p110-induced ERα-mediated transcription but had no effect on constitutively activated AKT2-stimulated ERα activity (Fig. 2*B*).

Moreover, we have observed that exogenous expression of ERα in ERα-positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2*C*), implying that ERα might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ERα-mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ERα Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ERα activation. ERα-positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2*D*). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ERα-mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

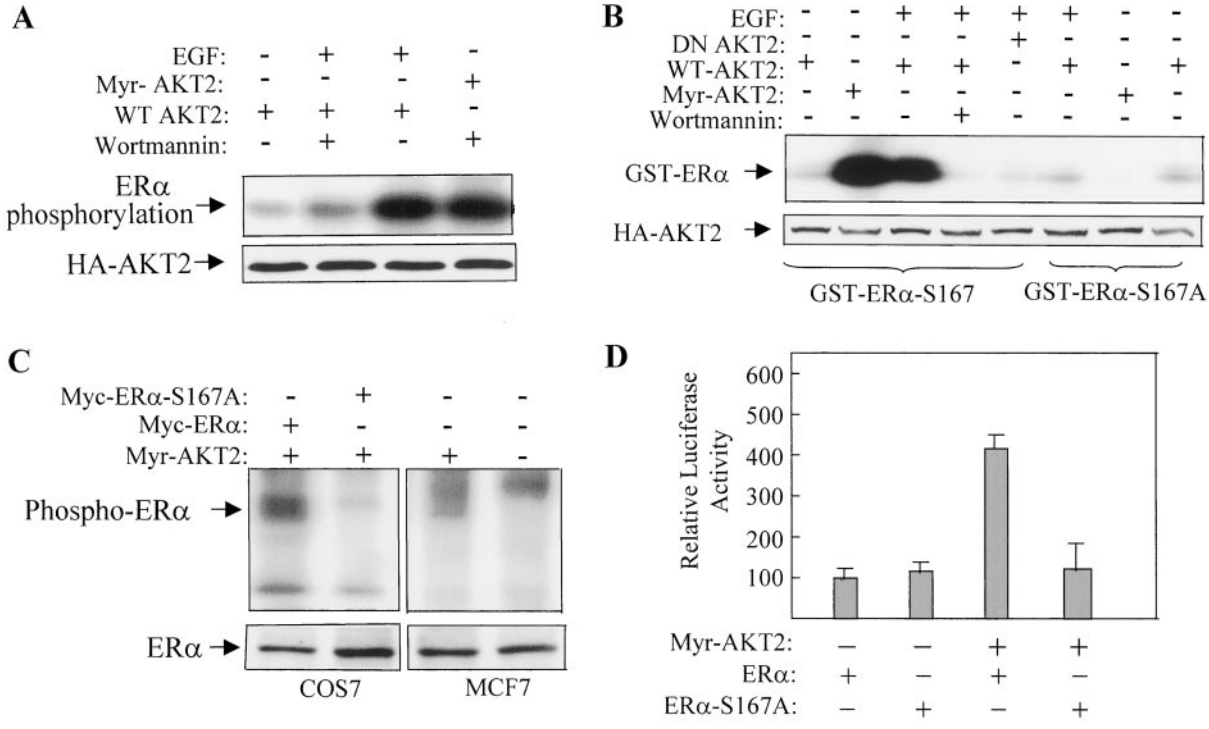

Fig. 3. AKT2 phosphorylates ERα on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ERα (*A*), GST-ERα-S167, and GST-ERα-S167A (*B*) were used as substrates. *C*, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with $[^{32}P]P_i$ for 4 h. Immunoprecipitates were prepared with anti-myc (*left*) or anti-ERα (*right*) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (*top*) and detected with anti-ERα antibody (*bottom*). *D*, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ERα transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ERα, or ERα-S167A, β-galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ERα by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and $pp90^{rsk1}$.

AKT2 Phosphorylated Serine-167 of ERα *in Vitro* and *in Vivo*. Phosphorylation of ERα has been shown to be an important mechanism by which ERα activity is regulated. ERα is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and $pp90^{rsk1}$/casine kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ERα *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ERα as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hERα. The ERα phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3*A*).

To determine whether AKT2 phosphorylates ERα *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with $[^{32}P]P_i$. The cell lysates were incubated with anti-ERα antibody, and the immunoprecipitates were separated on SDS-PAGE. ERα was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3*C*). These data indicate that AKT2 phosphorylated ERα both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ERα, possibly through the phosphorylation of serine residues. There are four serine residues (Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ERα protein sequence and found that serine-167 (^{162}**RER**LAS^{167}) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ERα-S167 but not GST-ERα-S167A mutant (Fig. 3*B*). The EGF-induced AKT2 phosphorylation of ERα is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ERα expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with $[^{32}P]P_i$ and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3*C*, constitutively active AKT2 phosphorylated wild-type ERα but not the ERα-S167A mutant *in vivo*, suggesting that serine-167 of ERα is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ERα transcriptional activity. To further examine whether AKT2-activated ERα transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ERα-S167A or wild-type ERα. Fig. 3*D* shows that ERα-S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ERα-mediated transcription through phosphorylation of serine-167.

ERα Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ERα binds to the p85α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

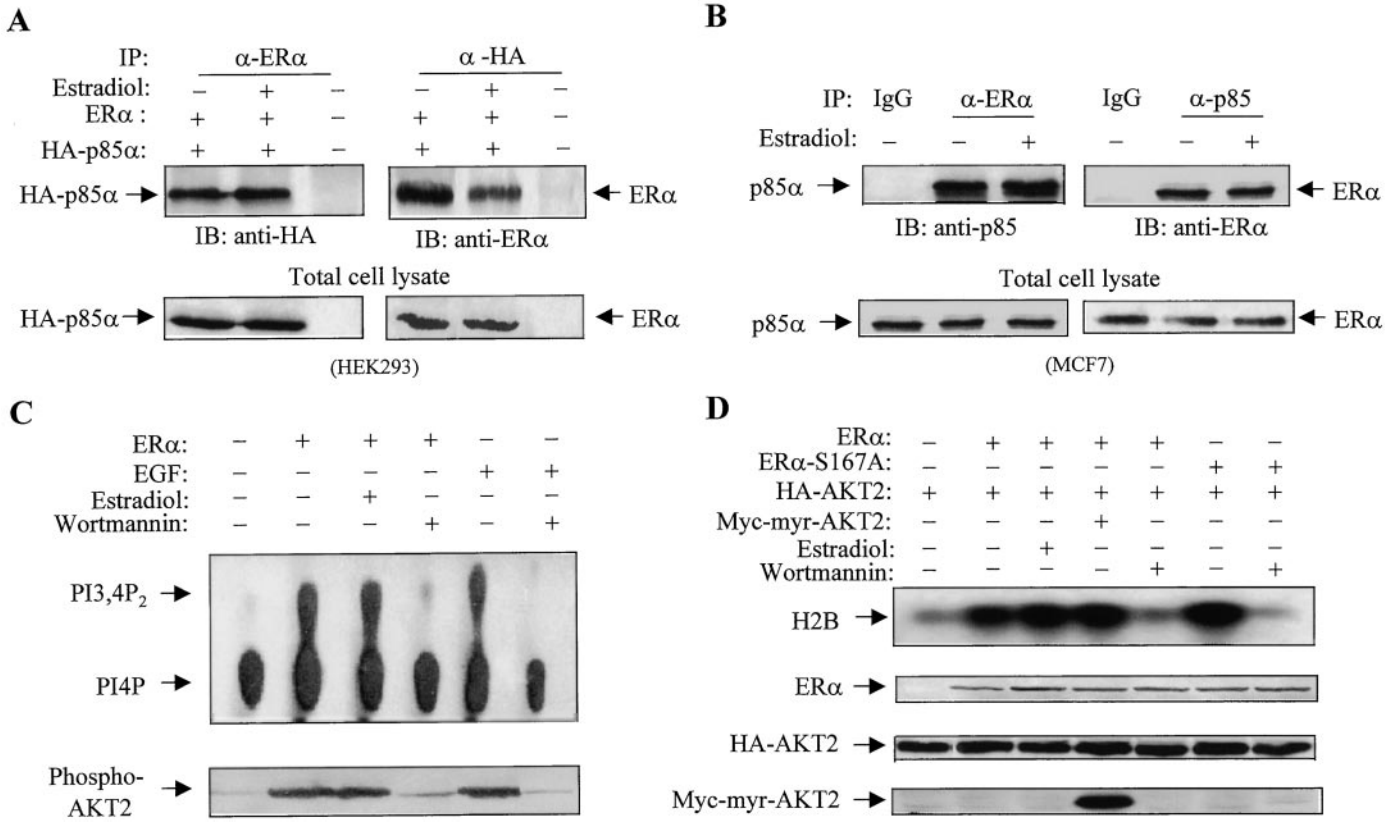

Fig. 4. ERα interacts with p85α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ERα and p85α in (*A*) HEK293 cells cotransfected with HA-p85α/ERα and in (*B*) nontransfected MCF7 cells. *Top*, coimmunoprecipitation; *bottom*, Western blot of total cell lysates. *C*, *in vitro* PI3K assay (*top*) of HEK293 cells transfected and treated with indicated plasmid and agents. *Bottom*, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. *D*, *in vitro* kinase assay (*top*) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. *Panels 2–4*, Western blots of transfected HEK293 cell lysates detected with anti-ERα, anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ERα failed to bind and activate PI3K, indicating that ERα-associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ERα binds to and activates PI3K/AKT2 in epithelial cells. ERα/HA-p85α-transfected HEK293 and nontransfected ERα-positive MCF7 cells were immunoprecipitated with anti-ERα and detected with anti-HA or anti-p85α antibody or *vice versa*. As shown in Fig. 4*A* and *B*, ERα constitutively associated with p85α, and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ERα in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4*C*). These data suggest that ERα binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ERα activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ERα-negative HEK293 cells were transfected with ERα or ERα-S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ERα significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ERα-induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ERα-S167A activated AKT2 at a similar level to that of wild-type ERα. Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ERα had the same effect on wild-type AKT2 activation as that of expression of ERα alone (Fig. 4*D*). These results indicate that activation of AKT2 by ERα is through PI3K and independent of ERα phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ERα transcriptional activity by phosphorylation of serine-167 *in vitro* and *in vivo*, and ERα activates PI3K/AKT2 kinase by binding to p85α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

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