P21-Activated Kinase 1 Regulation of Estrogen Receptor-α Activation Involves Serine 305 Activation Linked with Serine 118 Phosphorylation

Suresh K. Rayala,[1] Amjad H. Talukder,[1] Seetharaman Balasenthil,[1] Robbin Tharakan,[3] Christopher J. Barnes,[1] Rui-An Wang,[1] Marcelo Aldaz,[2] Sohaib Khan,[3] and Rakesh Kumar[1]

Departments of [1]Molecular and Cellular Oncology and [2]Carcinogenesis, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; and [3]Department of Cell Biology, Neurobiology, and Anatomy, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, Ohio

Abstract

Here, we investigated the role of P21-activated kinase 1 (Pak1) signaling in the function of estrogen receptor-α (ER-α) as assessed by serine 305 (S305) activation and transactivation activity of ER. We found that Pak1 overexpression interfered with the antiestrogenic action of tamoxifen upon the ER transactivation function in hormone-sensitive cells. In addition, tamoxifen stimulation led to up-regulation of ER target genes in breast cancer cells with increased Pak1 expression. Tamoxifen also increased Pak1-ER interaction in tamoxifen-resistant but not in tamoxifen-sensitive cells. Results from the mutational studies discovered a role of ER-S305 phosphorylation in triggering a subsequent phosphorylation of serine 118 (S118), and these effects were further potentiated by tamoxifen treatment. We found that S305 activation-linked ER transactivation function requires a functional S118, and active Pak1 signaling is required for a sustaining S118 phosphorylation of the endogenous ER. All of these events were positively influenced by tamoxifen and thus may contribute toward the loss of antiestrogenic effect of tamoxifen. These findings suggest that Pak1 signaling-dependent activation of ER-S305 leads to an enhanced S118 phosphorylation presumably due to a conformational change, and such structural modifications may participate in the development of tamoxifen resistance. (Cancer Res 2006; 66(3): 1694-701)

Introduction

The process of breast cancer progression requires, among other steps, changes in cytoskeleton signaling pathways, increased directional motility, and enhanced cell survival. P21-activated kinase 1 (Pak1), an effector of activated Rho GTPases Cdc42 and Rac1, is essential for a variety of cellular functions, including cell morphogenesis, cell motility, cell survival, angiogenesis, and mitosis (1, 2). In addition to cell motility, increased Pak1 activity correlates well with the invasiveness of human breast cancer cells and tumors (3–5). Adam et al. (6) have shown a mechanistic role for Pak1 activation in the increased cell invasion of breast cancer cells by growth factors. Furthermore, expression of a kinase-dead Pak1 mutant in the highly invasive breast cancer cell lines led to stabilization of stress fibers, enhanced cell spreading, and reduction in invasiveness (7). Conversely, conditional expression of kinase-active T423E Pak1 in the noninvasive MCF-7 breast cancer cells promoted cell migration and anchorage-independent growth (8). Pak1 activation also promotes cell survival by inactivating the proapoptotic proteins Bad (9), FKHR (10), and BimL (11), suggesting that Pak1 may be involved in cancer progression. Further, *Pak1* gene amplification and Pak1 protein up-regulation has been also reported in ovarian (12) and breast (3, 8) cancers. Consistent with these findings, hyperstimulation of Pak1 transgene in mammary glands induces hyperplasia as well as estrogen-inducible genes in the mammary epithelium (13).

The development of human breast cancer is also promoted by estrogen stimulation of mammary epithelial cell growth. The biological effects of estrogen are mediated by two distinct estrogen receptors (ERα and ERβ), which belong to the nuclear hormone receptor superfamily. ER is composed of six functionally distinct domains, termed A to F. Transactivation is mediated by two regions designated activation function-1 (AF-1) and AF-2. AF-1 is located at the amino-terminal A/B domain and functions in a ligand-independent manner. In contrast, AF-2 is located in the carboxyl-terminal E domain and acts in a ligand-dependent manner. Both AF-1 and AF-2 can activate transcription separately or together for full ER activity (14, 15). Upon estrogen binding, ER undergoes major conformational changes resulting in receptor dimerization and binding to specific DNA sequences termed estrogen response elements (ERE) in the promoter regions of the estrogen-responsive genes. The change in receptor conformation allows for the recruitment of coactivators and facilitates target gene transcription. Posttranslational modification, such as phosphorylation, further regulates ER activity. For example, serine 118 (S118) represents one of the major phosphorylation sites in the AF-1 domain and could be activated *in vivo* in a ligand- and mitogen-activated protein kinase–independent manner (16). Evidence also supports the notion that the growth of ER-positive breast cancer cells involves growth factor–triggered signaling cascade. For example, the human epidermal growth factor receptors family of receptors have been shown to regulate the proliferation of breast cancer cells (17), phosphorylate S118 in the AF-1 domain (18), and play an active role in the progression of breast cancer to a hormone-independent state. Likewise, growth factor–activated signaling kinases, such as Pak1, could phosphorylate serine 305 (S305) in the AF-2 domain and promote transactivation functions in the absence of ligand (13, 19). Interestingly, both S118 and S305 sites have been implicated in modifying the action of tamoxifen (20, 21), an antagonist in the

Requests for reprints: Rakesh Kumar, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-745-3558; Fax: 713-745-3792; E-mail: rkumar@mdanderson.org.

doi:10.1158/0008-5472.CAN-05-2922

breast, and, thus, transactivation functions of AF-2 and AF-1 domains are widely believed to be influenced by cross talk between growth factors and ER.

Because Pak1 is one of the major downstream signaling nodules of growth factors (1, 2), and because upstream Pak1 activators, such as AND-34/BCAR3 (22) and Rac3 (23), have been shown to modulate hormone sensitivity in the absence of estrogen, we initially set out to explore the hypothesis that Pak1 signaling might constitute an important regulator of ER transactivation. Here, we provide novel insights about the effect of Pak1 signaling on the transactivation and associated modifications of the activation function domains of ER.

Materials and Methods

Antibodies and reagents. Antibodies used for Western blotting, immunoprecipitation, and immunohistochemistry were as follows: ERα (Upstate Biotech, Lake Placid, NY), T7-Ab (Novagen, Milwaukee, WI), Pak1 and phospho-ER-S118 (Cell Signaling, Beverly, MA), phospho-ER-S305 (Bethyl Laboratories, Montgomery, TX), HA-tag (Boehringer Mannheim, Indianapolis, IN), myc-tag (BD Biosciences, San Jose, CA), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), actin and vinculin (Sigma Chemical Co., St. Louis, MO). Antimouse- and antirabbit-horseradish peroxidase (HRP) conjugates were from Amersham (Piscataway, NJ). Fluorescently conjugated secondary antibodies and the DNA counter stain ToPro3 were from Molecular Probes.

Cell culture, reporter assay, cell growth, and RNA interference. MCF-7, ZR75, Ishikawa, HeLa, and MDA-MB-231 cells were maintained in DMEM/F12 (1:1) supplemented with 10% FBS. MCF-7/tamoxifen-resistant (MCF-7/TAMR1) and Tam-sensitive (MCF-7/TAM-Sen) cells were maintained in 5% minimal essential medium, without phenol red containing 5% serum and the characterization of these cells has been previously described (24). MCF-7 Tet-on cells expressing dominant active T423E-Pak1(MCF-7/DA-Pak1 cells) were maintained in 5% tetracycline-free sera in RPMI (8). For cell growth assay, cells were grown in phenol red–free medium supplemented with 5% DCC in six-well plates, treated with or without tamoxifen (10^{-8} mol/L). Cells were trypsinized and counted with a Coulter counter at the indicated times.

Reporter gene assays. Transient transfection studies were done as previously described (11, 13) using FuGENE 6 according to the instructions of the manufacturer (Roche Applied Science, Indianapolis, IN). Cells were then lysed with passive lysis buffer, and the luciferase assay was done using a luciferase reporter assay kit (Promega). The total amount of DNA used in the transfections was kept constant by adding a parental vector. Each transfection was carried out in triplicates in six-well plates.

Short interfering RNA. Pak1 short interfering RNA (siRNA) was purchased from Cell Signaling and nonspecific random siRNA from Dharmacon (Lafayette, CO). siRNA transfections were carried out using Oligofectamine (Invitrogen), according to the protocol of the manufacturer, in six-well plates. After 48 hours, cells were prepared for luciferase assay and cell growth.

Cell extracts, immunoblotting, and immunoprecipitation. To prepare cell extracts, cells were washed thrice with PBS and then lysed in radioimmune precipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail (Roche Applied Science), and 1 mmol/L sodium vanadate] for 15 minutes on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 minutes. Cell lysates containing an equal amount of protein (~200 μg) were then resolved on an SDS-polyacrylamide gel (8% acrylamide), transferred to a nitrocellulose membrane, probed with the appropriate antibodies, and developed using either the enhanced chemiluminescence or alkaline phosphatase–based color reaction method. Cell lysates for immunoprecipitation were prepared using NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.5% NP40, 1× protease inhibitor cocktail, and 1 mmol/L sodium vanadate]. Immunoprecipitation was done for 4 hours at 4°C using 1 μg of antibody per milligram of protein (3, 11).

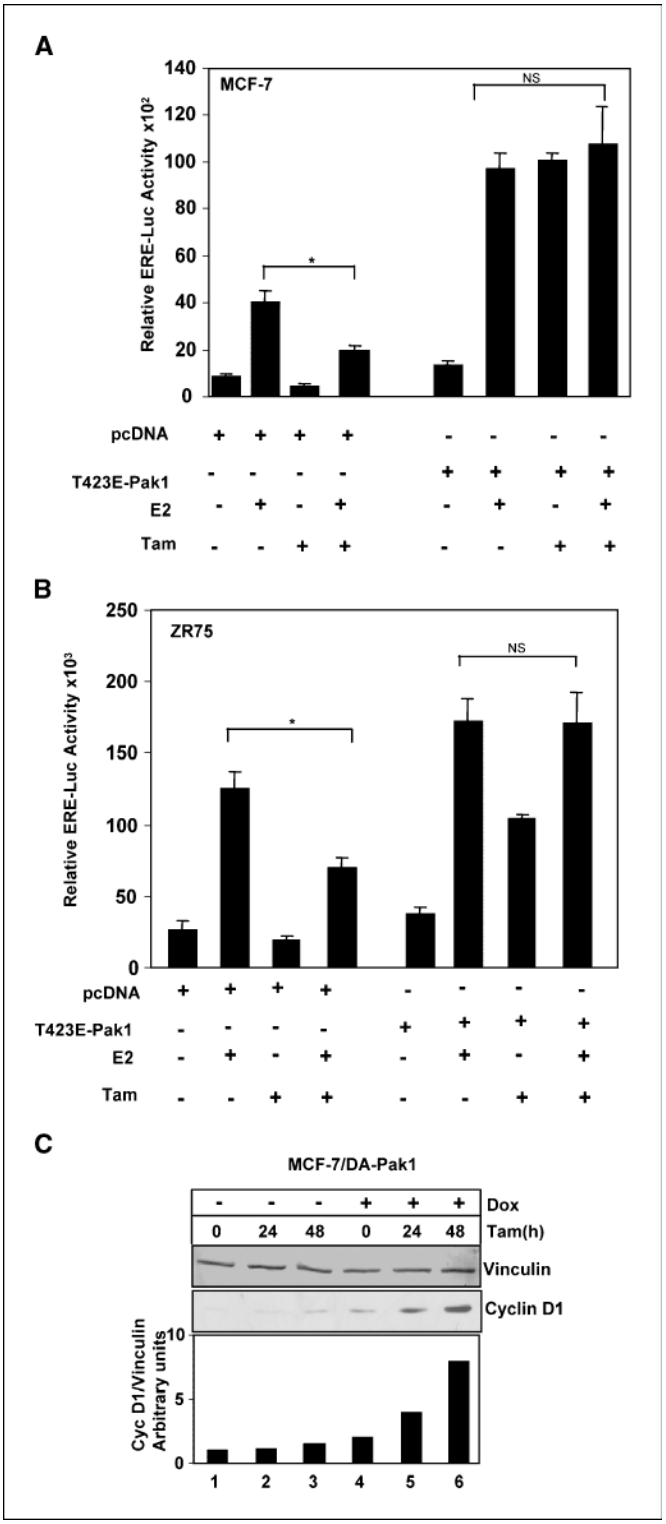

Figure 1. Pak1 activation inhibits tamoxifen action. *A* and *B,* MCF-7 and ZR75 cells were cotransfected with *ERE-luc* reporter gene along with cytomegalovirus or CMV-T423E-Pak1 expression vector. After 24 hours, cells were treated with or without E2 (10^{-9} mol/L) or tamoxifen (*Tam*, 10^{-8} mol/L), or both. Luc activity was measured after 24 hours. Similar results were obtained in three independent experiments. *Columns,* mean; *bars,* SE. *, significant *P* value; *NS,* nonsignificant (Student's *t* test). *C,* up-regulation of E2 responsive genes by tamoxifen. MCF-7/DA-Pak1 cells were treated with or without doxycycline (*Dox*, 1 μg/μL), then treated with tamoxifen at indicated time points. E2 responsive gene *cyclin D1* was analyzed by Western blotting.

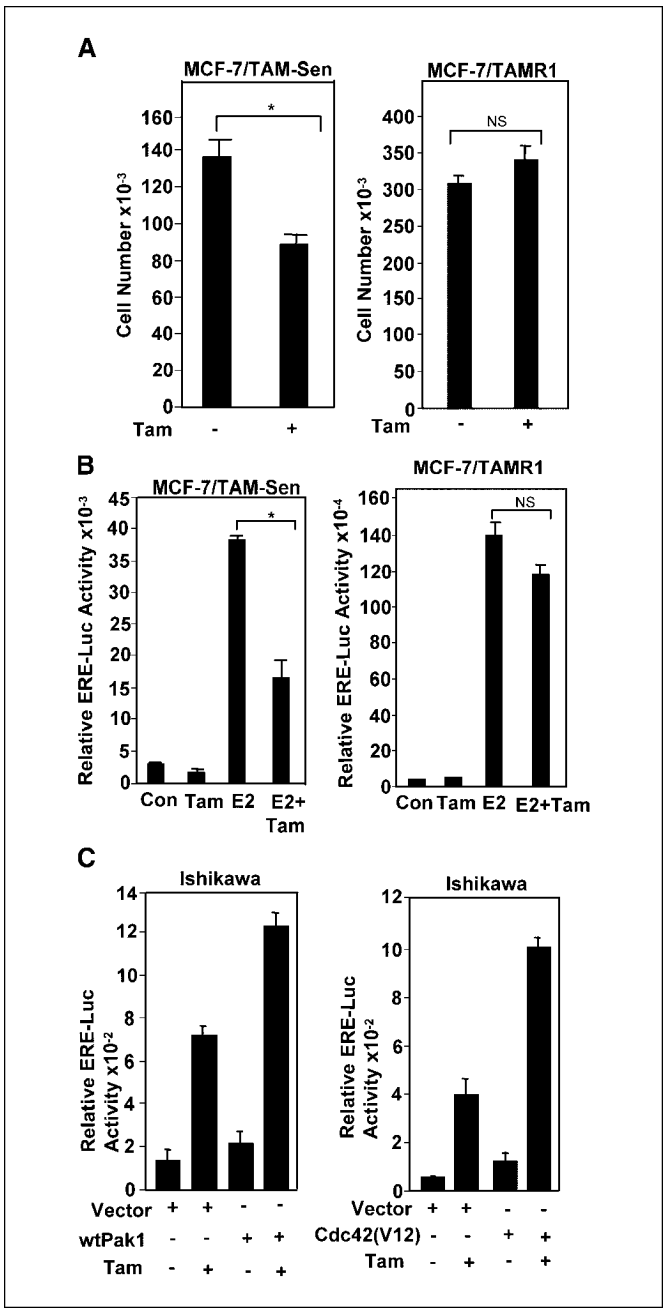

Figure 2. *A,* characterization of MCF-7/TAM-Sen and MCF-7/TAMR1 cells. MCF-7/TAM-Sen and MCF-7/TAMR1 cells were treated with or without tamoxifen (10^{-8} mol/L) for 5 days, and the cell number was determined. *B,* MCF-7/TAM-Sen and MCF-7/TAMR1 cells were transfected with ERE-luc and treated with E2 or tamoxifen for 24 hours and ERE-luc activity was measured. *, significant *P* value (Student's *t* test). *C,* tamoxifen potentiated Pak1 regulation of ER-transactivation in Ishikawa cell line and potentiated activated Cdc42 (V12)–mediated ERE-luc activity in Ishikawa cells. *Columns,* mean; *bars,* SE.

Pak1 kinase assay. Pak1 kinase assay using myelin basic protein was done as described (8). Briefly, Ishikawa cells were immunoprecipitated with Pak1 antibody. Immunocomplexes were washed thrice with NP40 lysis buffer and twice with kinase buffer containing 20 mmol/L HEPES (pH 7.4), 1 mmol/L DTT, 10 mmol/L $MnCl_2$, and 10 mmol/L $MgCl_2$. The kinase reaction was carried out in kinase buffer supplemented with myelin basic protein as a substrate and 10 μCi of [γ^{32}P] ATP at 30°C for 30 minutes. The kinase reaction was terminated by the addition of 4× SDS-PAGE sample buffer, run on SDS-PAGE, and followed by autoradiography.

Plasmid construction. Wild-type (WT) myc-tagged Pak1; catalytically active Pak1 T423E constructs (8); and WT-ER, ER-305E, and ER305A (13) were described previously. Site-directed point mutations for ER-S118A and ER 305E118A were done by using Quick Change Site Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) according to the instructions of the manufacturer.

Transgenic studies. Generation of transgenic mice expressing constitutively kinase-active T423E Pak1 has been previously described (13).

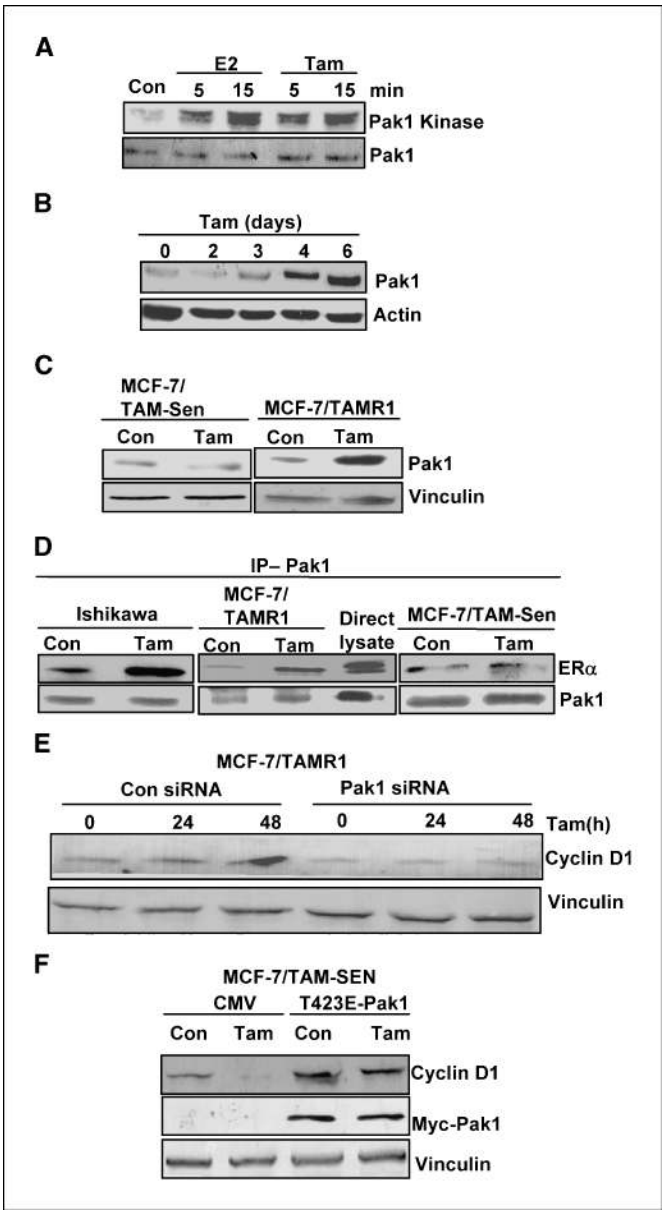

Figure 3. Tamoxifen regulation of Pak1 expression and activity. *A,* up-regulation of Pak1 activity in Ishikawa cells by tamoxifen and E2. *B,* up-regulation of Pak1 expression in Ishikawa cells by tamoxifen. *C,* up-regulation of Pak1 expression in MCF-7/TAMR1 cell line by treatment with tamoxifen for 24 hours. *D,* tamoxifen enhanced Pak1-ER interaction in Ishikawa and MCF-7/TAMR1 cells. Cells were treated with or without tamoxifen (10^{-8} mol/L) for 24 hours and immunoprecipitated with anti-Pak1 antibody and immunoblotted with ERα or Pak1 antibodies. *E,* tamoxifen up-regulation of cyclin D1 is compromised by Pak1 knockdown in MCF-7/TAMRI cells. Cells were transfected with control or Pak1-specific siRNA. After 48 hours, cells were treated with tamoxifen (10^{-8} mol/L) and cyclin D1 protein levels were analyzed by Western blotting. *F,* transient overexpression of catalytically active Pak1 (Pak1-T423E) in MCF-7/TAM-Sen cells suppressed the ability of tamoxifen to down-regulate cyclin D1 expression.

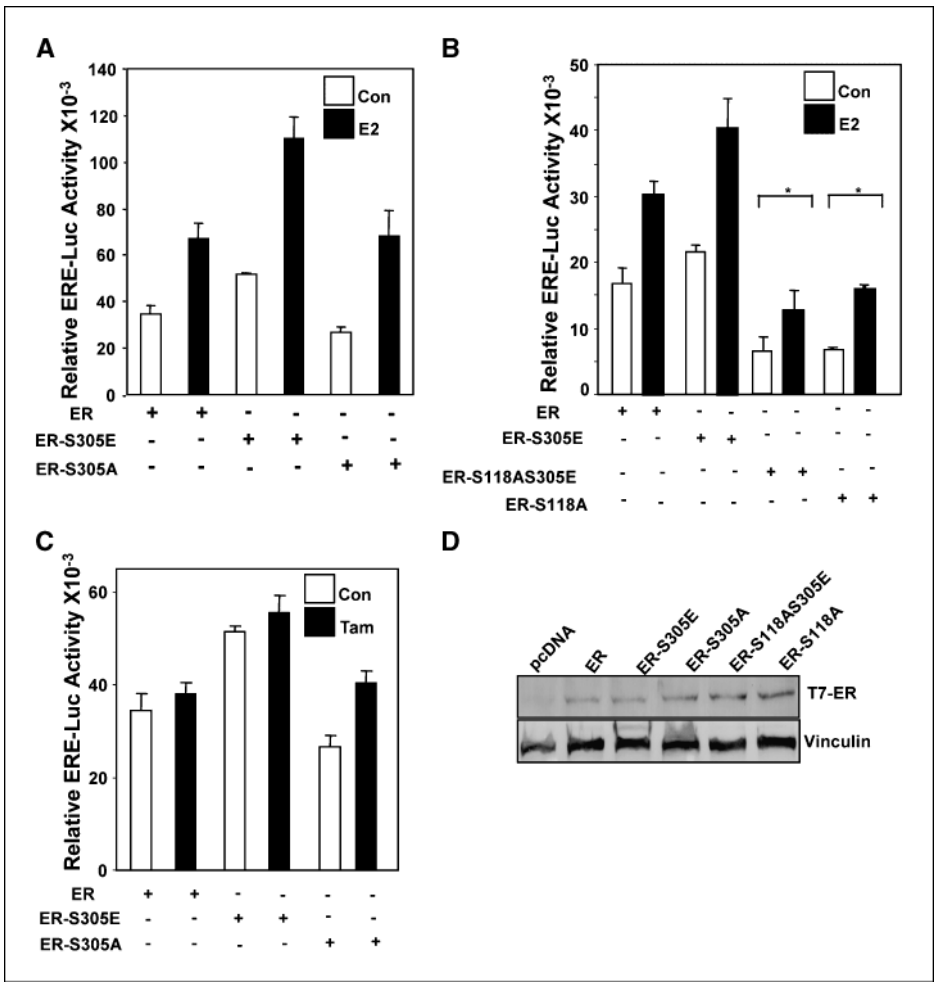

Figure 4. Transactivation activity of ER-S305 mutants. *A,* HeLa cells were cotransfected with 200 ng ERE-luciferase reporter, 500 ng ER, ER-S305E, or ER-S305A with or without 10^{-9} mol/L E2 stimulation. S305E mutation of ER resulted in stimulation of transactivation activity in the absence of estrogen treatment and was further increased upon 10^{-9} mol/L E2 treatment. *Columns,* mean; *bars,* SE. *B,* HeLa cells were cotransfected with 200 ng of ERE-luciferase reporter, 500 ng of ER, ER-S305E, or ER-S118AS305E with or without 10^{-9} mol/L E2 stimulation. Mutation of ER-S118AS305E blocked the ERE-luc transactivation by S305E, as S118A. *Columns,* mean; *bars,* SE. *, significant *P* value. *C,* HeLa cells were cotransfected with 200 ng of ERE-luciferase reporter, 500 ng of ER, ER-S305E, or ER-S305A with or without 10^{-8} mol/L tamoxifen stimulation. No further significant increase in transactivation was observed following upon tamoxifen treatment. *Columns,* mean; *bars,* SE. *D,* HeLa cells were transfected with 1 μg ER, ER-S305E, ER-S305A, ER-S118A305E, or ER-S118A, and expression of these constructs was analyzed by Western blotting using T7 antibody.

Immunohistochemistry. Immunohistochemistry using paraffin-embedded sections was done as described (3). Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized with xylene and rehydrated using graded ethanol. Sections were incubated in 0.3% hydrogen peroxide and methanol for 30 minutes to inactivate the endogenous peroxidase. The sections were then boiled for 10 minutes in 0.01 mol/L citrate buffer and cooled for 30 minutes at room temperature to expose antigenic epitopes. The sections were blocked with 2% normal goat serum in 1% bovine serum albumin (BSA) and PBS for 30 minutes and then incubated overnight at room temperature with primary antibody against ER-S118, 1:25 dilution (Cell Signaling) and ER-S305 1:25 dilution (Bethyl Laboratories). Primary antibodies were diluted in dilution buffer (2% normal goat serum, 1% BSA, and PBS). The sections were washed thrice with 0.05% Tween in PBS for 10 minutes, incubated with HRP secondary antibody (1:100 dilution; Amersham) for 1 hour; and then washed thrice with 0.05% Tween in PBS for 10 minutes. The sections were then developed with diaminobenzidine-H_2O_2 and counterstained with Mayer's hematoxylin.

Immunofluorescence. The cellular localization of ER-S118 and ER-S305 was determined using indirect immunofluorescence as previously described (25). Briefly, MCF-7 Tet-on cells expressing dominant active T423E-Pak1 (MCF-7/DA-Pak1 cells) were grown on glass coverslips and treated as described. Cells were then fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4), with 0.1% Triton X-100 permeabilization (ER-S305) or in 100% methanol (ER-S118), then incubated with specific primary antibodies followed by Alexa-546–labeled goat anti-rabbit antibody (red) or Alexa-488–labeled goat anti-mouse antibody (green). DNA was localized using ToPro3 (blue). Fluorescent labeling was visualized using an Olympus FV300 laser scanning confocal microscope with a ×60 objective and PC-based Olympus Fluoview software.

Statistical analysis. Statistical analysis was done using Student's *t* test and values with $P < 0.05$ were considered statistically significant.

Results

Pak1 overexpression inhibits tamoxifen action. Because recent studies have shown that Pak1 stimulates ER-driven transactivation in breast cancer cells (13, 19), we hypothesized that Pak1 regulation of ER may modulate the action of tamoxifen in breast cancer cells. We first determined the likelihood that Pak1 up-regulation may modify tamoxifen sensitivity and examined the effect of tamoxifen in antagonizing ER transcription and the suppressive effect of Pak1. We found that transient overexpression of catalytically active Pak1 (Pak1-T423E) in tamoxifen-sensitive MCF-7 and ZR75 cells leads to inhibition of antiestrogenic action of tamoxifen and allows tamoxifen to transactivate ERE-driven reporter gene (Fig. 1*A* and *B*). In support of the observed suppression of antiestrogenic action of tamoxifen by Pak1 overexpression, we used a previously characterized MCF-7 clone (MCF-7/DA-Pak1 cells) expressing catalytically active Pak1 under an inducible Tet-on promoter (8). We examined whether conditional overexpression of Pak1 also affects the ability of tamoxifen to modulate ER target genes in MCF-7/DA-Pak1 cells. Results showed that Pak1 overexpression in breast cancer cells results in increased ability of

tamoxifen to stimulate the expression of an ER-regulated gene such as *cyclin D1* (Fig. 1*C*).

To directly validate the above possibility, we used recently generated MCF-7 clones that are either sensitive (MCF-7/TAM-Sen) or resistant (MCF-7/TAMR1) to tamoxifen (24), as measured by the growth-rate (Fig. 2*A*) or ERE-luc activity (Fig. 2*B*). We next examined the potential relationship between Pak1 hyperactivation and tamoxifen insensitivity (inability of tamoxifen to inhibit estrogen-induced transactivation) in human endometrial cancer cell line Ishikawa. Tamoxifen does not inhibit the ligand-induced cellular response in Ishikawa cells. We found that transient expression of Pak1 in Ishikawa cells increased the ability of tamoxifen to stimulate ERE-dependent transactivation and cooperated with tamoxifen in this process (Fig. 2*C*). This observed effect of Pak1 in Ishikawa cells was also mimicked by an activated form of its upstream regulator Cdc42 (Fig. 2*C*). These results suggest that Pak1 activation might be closely linked with the partial inhibition of antiestrogenic action of tamoxifen in hormone-sensitive cells.

Tamoxifen promotes Pak1 expression and Pak1-ER interaction. Because both Pak1 and tamoxifen up-regulate ER transactivation (Fig. 1), we next explored whether tamoxifen in turn influences Pak1 activity. Unexpectedly, tamoxifen, like estrogen, also up-regulated Pak1 activity in Ishikawa cells (Fig. 3*A*). Because tamoxifen insensitivity is acquired over time, we next determined the long-term effect of tamoxifen on the Pak1 pathway in Ishikawa cells. Tamoxifen treatment was accompanied by an increase in Pak1 protein expression in Ishikawa cells (Fig. 3*B*). Likewise, tamoxifen also induced Pak1 protein expression in MCF-7/TAMR1 cells but not in MCF-7/TAM-Sen cells (Fig. 3*C*). These results suggest that tamoxifen-stimulated Pak1 expression leads to persistent Pak1 activity and, consequently, in the maintenance of tamoxifen-insensitive phenotypes.

Because Pak1 interacts with and phosphorylates ERα at S305 and stimulates ER transactivation, we next examined the effect of tamoxifen on the interaction of Pak1 and ER. Coimmunoprecipitation studies showed that tamoxifen promoted the interaction of the endogenous Pak1 with ER in tamoxifen-insensitive Ishikawa and MCF-7/TAMR1 cells, but not in MCF-7/TAM-Sen cells (Fig. 3*D*).

To show a mechanistic role for Pak1 in the stimulation of ER transactivation by tamoxifen, we knocked down Pak1 in MCF7/TAMR1 cells using Pak1-specific siRNA and analyzed for expression of the ER-regulated gene, *cyclin D1*. As shown in Fig. 3*E*, transient expression of Pak1-siRNA substantially reduced the ability of tamoxifen to stimulate *cyclin D1* expression compared with the cells treated with control siRNA. Comparatively, transient overexpression of catalytically active Pak1-T423E in MCF7/TAM-Sen cells suppressed the ability of tamoxifen to down-regulate cyclin D1 expression compared with control vector–transfected cells. However, we did not notice any change in the levels of cyclin D1 with Pak1 and tamoxifen, which could be due to transient expression (Fig. 3*F*). Together, these findings suggested a role of Pak1 overexpression in supporting antiestrogenic action of tamoxifen.

S305-associated ER transactivation activity requires a functional S118. Because overall transcription activity of ER is a result of both activation function domains (20, 21) and because S118 is activated in both ligand-dependent and ligand-independent manner (16), we next determined the potential significance of S118 in the transactivation activity of ER with an activated S305 wherein S305 was mutated to the acidic glutamic acid (S305E) or alanine (S305A). Results show that expression of S305E, but not S305A, potentiated ER-dependent transactivation from an ERE-based reporter both in the presence or absence of estrogen (Fig. 4*A*), whereas S118A (ER-S118A) mutation in the context of S305E (ER-S118A, S305E) significantly inhibited the basal transcription, as ER-S118A (Fig. 4*B*). Treatment with tamoxifen had no significant effect on ER-S305E–dependent transcription from ERE reporter (Fig. 4*C*). The expression profile of all the ERα constructs is shown in Fig. 4*D*.

ER activation on S305 leads to constitutive phosphorylation of S118. We next tested the hypothesis that the phosphorylation of ER on S305 could influence the activation status of S118. Exponentially growing HeLa cells were transfected with T7-ER or T7-ER-S305A, cell lysates were immunoprecipitated with an anti-T7-antibody and immunoblotted with antibodies against ER-S118 or ER. We found that ER-S305A was not phosphorylated at ER-S118 (Fig. 5*A*). Conversely, mutation of S305 to glutamic acid resulted in an increase of basal, estrogen- and tamoxifen-induced ER-S118 phosphorylation (Fig. 5*B* and *C*). These results suggested that Pak1 phosphorylation of ER on S305 leads to a secondary activation of S118.

Regulation of endogenous ER-S118 phosphorylation by Pak1 signaling. To directly establish the significance of Pak1 signaling on the status of ER-S118 phosphorylation, we next examined the effect of knockdown of endogenous Pak1 by Pak1-specific siRNA on the levels of ER-S118 phosphorylation. We found that Pak1 siRNA-mediated inhibition of Pak1 expression was accompanied by in-parallel reduction of ER-S118 phosphorylation with marginal effect on the levels of total ER protein in MCF-7 cells grown in 10% fetal bovine serum (Fig. 6*A*). It is interesting to note that Pak1 knockdown also blocked the estrogen induced ER-S118 phosphorylation (Fig. 6*B*). In contrast, transient coexpression of myc-tagged kinase-active T423E Pak1 and T7-tagged ER in HeLa cells elevated the amount of ER-S118 phosphorylation (Fig. 6*C*). To further implicate a regulatory role for Pak1 signaling in the regulation of ER-S118 phosphorylation, we next used MCF-7/DA-Pak1 cells (8), and, as

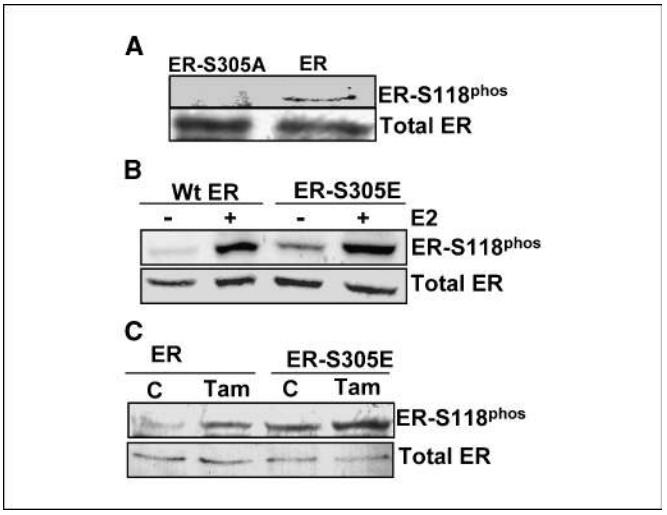

Figure 5. Transactivation activity of ER-S305 mutants and constitutive phosphorylation of S118. *A,* HeLa cells were transfected with ER or ER-S305A, immunoprecipitated with T7 antibody and probed with ER-phospho-S118 antibody and ER antibody. Transient expression of WT-ER but not ER-S305A resulted in significant phosphorylation of ER on S118. *B,* E2-induced phosphorylation of ER-S118 in HeLa cells transfected with WT-ER and ER-S305E. *C,* tamoxifen-induced phosphorylation of ER-S118 in MDA-MB-231 cells transfected with ERWT and ER-S305E.

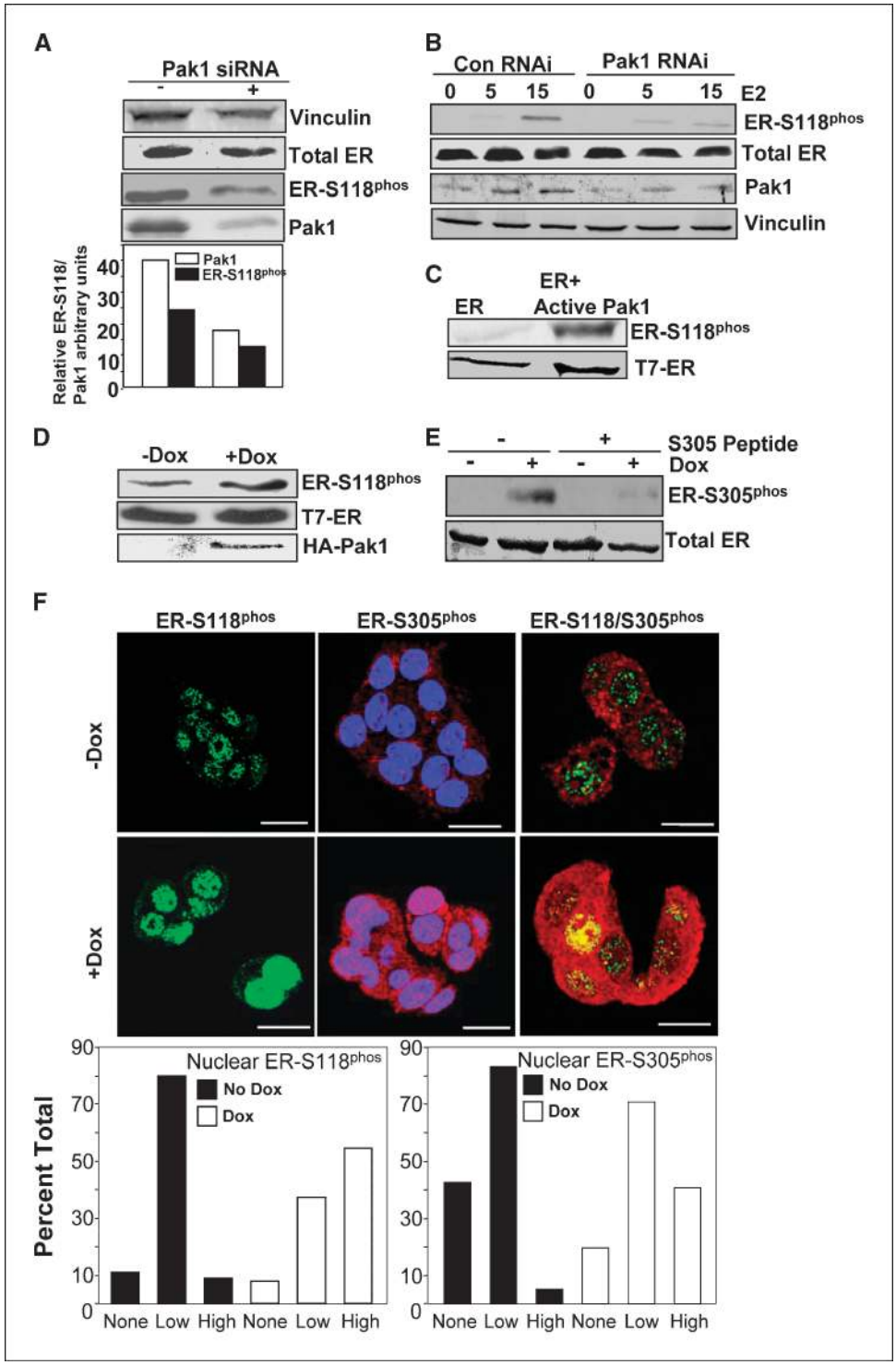

Figure 6. Pak1 regulation of ER-S118 phosphorylation. *A,* Pak1 knockdown leads to decreased ER-S118 phosphorylation. Exponentially growing MCF-7 cells were cotransfected with control siRNA or Pak1 siRNA, and cell lysates were immunoblotted with the indicated antibodies. *B,* Pak1 knockdown compromised estrogen-induced ER-S118 phosphorylation. *C,* up-regulation of ER-S118 phosphorylation by kinase-active T423E Pak1 in HeLa cells cotransfected with WT-ER. *D,* increased levels of ER-S118 phosphorylation in MCF-7-DA-Pak1 cells following induction with doxycycline. *E,* increased levels of ER-S305 phosphorylation in MCF-7/DA-Pak1 cells following induction with doxycycline and the same was blocked by using blocking peptide against ER-S305. *F,* immunofluorescent localization of ER-S305 phosphorylation (*red*) or ER-S118 phosphorylation (*green*) in MCF-7-DA-Pak1 cells before (*top*) and after (*bottom*) induction with doxycycline (1 μg/μL) for 24 hours. *Blue,* DNA counterstain. *Yellow,* colocalization. Bar, 20 μm.

expected, induced expression of HA-tagged T423E-Pak1 in MCF-7 cells stimulated the levels of ER-S118 phosphorylation as well as ER-S305 phosphorylation (Fig. 6*D, E* and *F*), with an increase in strong nuclear staining from 9% to 55% of cells for ER-S118 and of 4% to 31% for S305. In addition, ~50% of nuclei costained for ER-S118 and ER-S305 showed marked colocalization between the two phospho-antibodies (Fig. 6*F, right*). Recent work from our laboratory had shown that overexpression of a kinase-active T423E Pak1 transgene in the murine mammary gland leads to a widespread hyperplasia (13).

We next used this model to validate whether hyperactivation of Pak1 in the mammary gland was also accompanied by an increased ER-S305 and ER-S118 phosphorylation. Results from immunohistochemical studies indicated that, indeed, transgenic mammary glands from kinase-active Pak1 mice exhibited an intense nuclear staining of both activated ER-S305 and ER-S118 (Fig. 7*A* and *B*).

The specificity of ER-S305 staining was revealed by using a specific peptide against ER-S305 (Fig. 7*A*). Tissue lysates from WT and active Pak mice were analyzed for ER-S118 phosphorylation by Western blotting, which showed an increased ER-S118 phosphorylation in mammary gland tissue from kinase-active Pak1 mice (Fig. 7*C*).

Discussion

We undertook this study to understand the role of Pak1 in the transactivation function of ER and whether these functions are influenced by estrogen and by tamoxifen, by using breast and endometrial cancer cell lines and in transgenic mice expressing catalytically active Pak1 in mammary glands. Our results showed that activation of the Pak1 pathway interferes with the antiestrogenic action of tamoxifen in hormone-sensitive cells, thus finding a role of Pak1 hyperstimulation in conferring resistance to antiestrogenic action of tamoxifen. Consistent with such a notion, we noticed increased Pak1 activity in breast cancer cells with an acquired tamoxifen resistance and in endometrial cancer cells that are insensitive to tamoxifen. Conversely, conditional down-regulation of Pak1 in tamoxifen-resistant cells partially restored the antiestrogenic action of tamoxifen, whereas transient up-regulation of Pak1 inhibited antiestrogen effect of tamoxifen.

The finding that tamoxifen stimulates Pak1 expression and, in turn, increased Pak1 activity in tamoxifen-resistant cells is important as it raises the possibility of existence of distinct cellular mechanisms in tamoxifen-resistant cells, because tamoxifen was

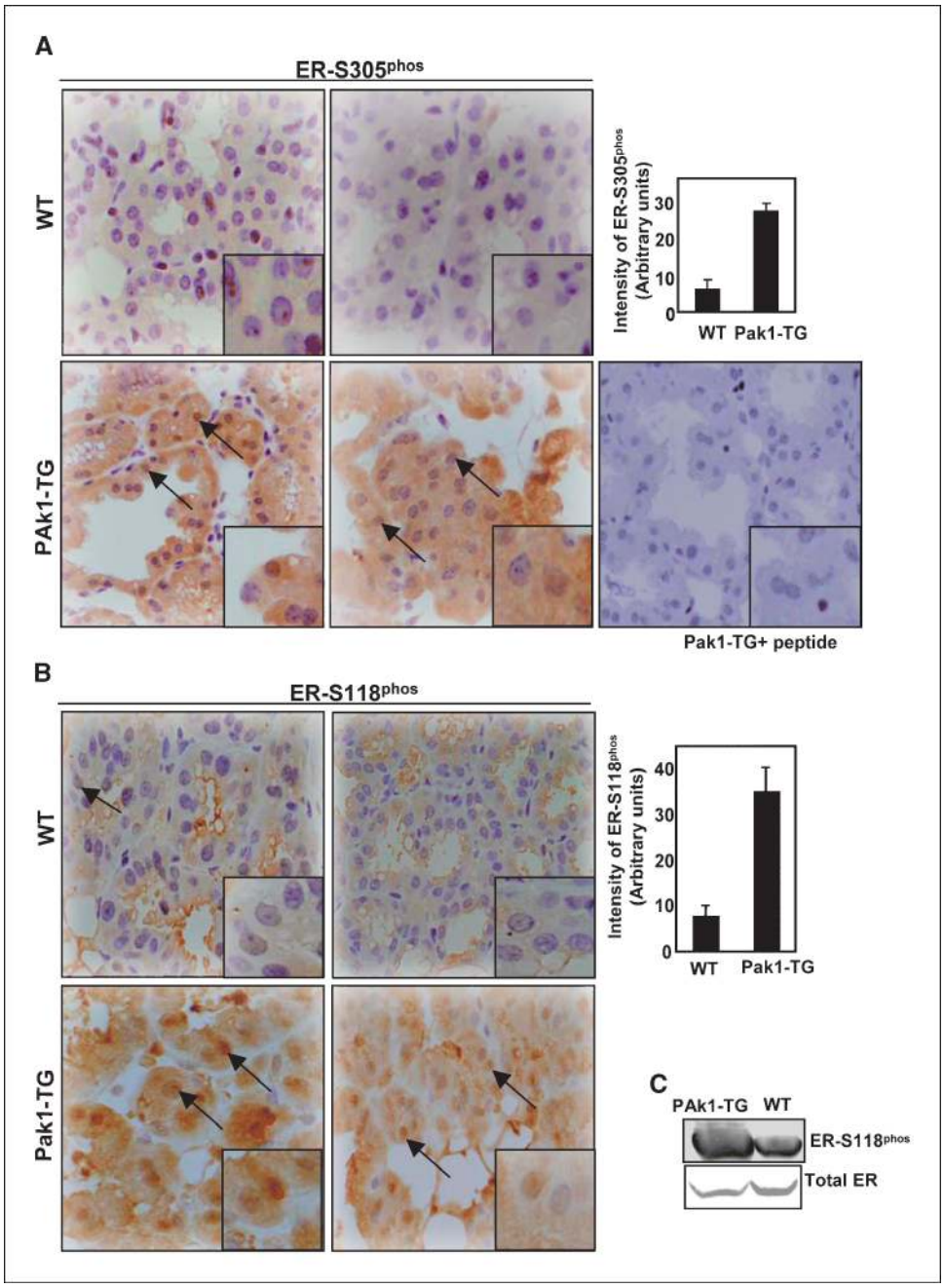

Figure 7. Activated Pak1 promotes ER stimulation of S305 and S118 phosphorylation in the transgenic mammary gland. *A* and *B,* tissue sections from mammary gland of kinase-active T423E Pak1 transgenic mice (*Pak1-TG*) showing increased ER-S305 phosphorylation (*A*) and ER-S118 phosphorylation (*B*), as analyzed by immunohistochemical staining (×40 magnification). *Arrows,* nuclear staining. Tissue sections from mammary gland of WT mice were used as control. *C,* tissue lysates from mammary gland of kinase-active T423E Pak1 transgenic mice showing increased ER-S118 phosphorylation as analyzed by Western blot analysis.

unable to induce Pak1 pathway in tamoxifen-sensitive cells. Accordingly, tamoxifen also promoted Pak1 interaction with ER only in tamoxifen-resistant cells. Because earlier studies have shown that Pak1 phosphorylates ER on S305 in a physiologic setting (13), it is possible that the noted tamoxifen-induced Pak1 activity and increased Pak1-ER interaction might lead to transcription activation of ER as shown here.

Another notable finding of this study is that the transcription potentiating function of S305 activation requires a functional S118. This finding is consistent with the previous studies suggesting a role of cooperative interactions of both activation function domains of ER in the overall transactivation activity of ER (20, 21). The observed activation of S118 was attributed, at least in part, to conformational changes triggered by S305 activation in the absence of any exogenous ligand. However, overall transcriptional activity of ER due to S305 activation was enhanced by tamoxifen treatment, probably due to a persistent active conformation of the AF-2 domain introduced by S305E mutation. In summary, we found that ER activation on S305 increases the levels of S118 phosphorylation and that these changes could be further potentiated by tamoxifen. Because we have used a constitutively activated form of ER-S305E, findings presented here suggest that S305 stimulation might confer a conformational change, which allows for a better interaction with ligands such as tamoxifen. In this context, it is worth mentioning that while this study was in progress, Michalides et al. (20) have shown that another signaling kinase protein kinase A can also phosphorylate S305 and leads to an active conformational arrest upon tamoxifen binding and, in turn, tamoxifen-induced transactivation. In the light of the results presented here, we now suggest a working model wherein the noted functionality of signaling kinase-dependent S305 activation result in enhanced S118 phosphorylation. Because growth factor signaling could activate Pak1, and because the consensus phosphorylation motif in the Pak substrates is not very restricted and common with other kinases such as protein kinase A (26) and ribosomal S6 kinase (27), these findings suggest a broader role of ER-S305 phosphorylation by growth factors in conferring tamoxifen-responsive phenotypes.

Acknowledgments

Received 8/16/2005; revised 11/5/2005; accepted 12/5/2005.

Grant support: NIH grants CA90970 and CA98823 (R. Kumar).

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.

References

1. Bokoch GM. Biology of the p21-activated kinases. Annu Rev Biochem 2003;72:743–81.
2. Kumar R, Vadlamudi RK. Emerging functions of p21-activated kinases in human cancer cells. J Cell Physiol 2002;193:133–44.
3. Balasenthil S, Sahin AA, Barnes CJ, et al. p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. J Biol Chem 2004; 279:1422–8.
4. Salh B, Marotta A, Wagey R, Sayed M, Pelech S. Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. Int J Cancer 2002;98:148–54.
5. Stofega MR, Sanders LC, Gardiner EM, Bokoch GM. Constitutive p21-activated kinase (PAK) activation in breast cancer cells as a result of mislocalization of PAK to focal adhesions. Mol Biol Cell 2004;15:2965–77.
6. Adam L, Vadlamudi R, Kondapaka SB, Chernoff J, Mendelsohn J, Kumar R. Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. J Biol Chem 1998;273:28238–46.
7. Adam L, Vadlamudi R, Mandal M, Chernoff J, Kumar R. Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. J Biol Chem 2000;275:12041–50.
8. Vadlamudi RK, Adam L, Wang RA, et al. Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. J Biol Chem 2000;275:36238–44.
9. Schurmann A, Mooney AF, Sanders LC, et al. p21-activated kinase 1 phosphorylates the death agonist Bad and protects cells from apoptosis. Mol Cell Biol 2000;20: 453–61.
10. Mazumdar A, Kumar R. Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells. FEBS Lett 2003;535:6–10.
11. Vadlamudi RK, Bagheri-Yarmand R, Yang Z, et al. Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. Cancer Cell 2004;5:575–85.
12. Schraml P, Schwerdtfeger G, Burkhalter F, et al. Combined array comparative genomic hybridization and tissue microarray analysis suggest PAK1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma. Am J Pathol 2003;63:985–92.
13. Wang RA, Mazumdar A, Vadlamudi RK, Kumar R. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-α and promotes hyperplasia in mammary epithelium. EMBO J 2002;21:5437–47.
14. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003;9: 1980–9.
15. Nilsson S, Gustafsson JA. Biological role of estrogen and estrogen receptors. Crit Rev Biochem Mol Biol 2002; 37:1–28.
16. Karas RH, Gauer EA, Bieber HE, Baur WE, Mendelsohn ME. Growth factor activation of the estrogen receptor in vascular cells occurs via a mitogen-activated protein kinase-independent pathway. J Clin Invest 1998;101:2851–61.
17. Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 2003;9:447–54S.
18. Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 1996;15:2174–83.
19. Balasenthil S, Barnes CJ, Rayala SK, Kumar R. Estrogen receptor activation at serine 305 is sufficient to upregulate cyclin D1 in breast cancer cells. FEBS Lett 2004;567:243–7.
20. Michalides R, Griekspoor A, Balkenende A, et al. Tamoxifen resistance by a conformational arrest of the estrogen receptor α after PKA activation in breast cancer. Cancer Cell 2004;5:597–605.
21. Murphy L, Cherlet T, Adeyinka A, Niu Y, Snell L, Watson P. Phospho-serine-118 estrogen receptor-α detection in human breast tumors *in vivo*. Clin Cancer Res 2004;10:1354–9.
22. Cai D, Iyer A, Felekkis KN, et al. AND-34/BCAR3, a GDP exchange factor whose overexpression confers antiestrogen resistance, activates Rac, PAK1, and the cyclin D1 promoter. Cancer Res 2003;63:6802–8.
23. Mira JP, Benard V, Groffen J, Sanders LC, Knaus UG. Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc Natl Acad Sci U S A 2000; 97:185–9.
24. Hodges LC, Cook JD, Lobenhofer EK, et al. Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res 2003;1:300–11.
25. Rayala SK, den Hollander P, Balasenthil S, Yang Z, Broaddus RR, Kumar R. Functional regulation of oestrogen receptor pathway by the dynein light chain 1. EMBO Rep 2005;6:538–44.
26. Howe AK, Juliano RL. Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. Nat Cell Biol 2000;2: 593–600.
27. Woo MS, Ohta Y, Rabinovitz I, Stossel TP, Blenis J. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. Mol Cell Biol 2004;24:3025–35.

P21-Activated Kinase 1 Regulation of Estrogen Receptor-α Activation Involves Serine 305 Activation Linked with Serine 118 Phosphorylation

Suresh K. Rayala, Amjad H. Talukder, Seetharaman Balasenthil, et al.

Cancer Res 2006;66:1694-1701.