

# Phosphorylation of Estrogen Receptor $\alpha$ Blocks Its Acetylation and Regulates Estrogen Sensitivity

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## ABSTRACT

Estrogen receptor (ER)  $\alpha$  is mutated (lysine 303 to arginine, K303R) in approximately one third of premalignant breast hyperplasias, which renders breast cancer cells expressing the mutant receptor hypersensitive for proliferation in response to low doses of estrogen. It is known that ER $\alpha$  is posttranslationally modified by protein acetylation and phosphorylation by a number of secondary messenger signaling cascades. The K303R ER $\alpha$  mutation resides at a major protein acetylation site adjacent to a potential protein kinase A (PKA) phosphorylation site at residue 305 within the hinge domain of the receptor. Mutation of this phosphorylation site to aspartic acid to mimic constitutive phosphorylation blocks acetylation of the K303 ER $\alpha$  site and generates an enhanced transcriptional response similar to that seen with the naturally occurring K303R mutant receptor. Activation of PKA signaling by the cell-permeable cyclic AMP (cAMP) analog 8-bromo-cAMP further enhances estrogen sensitivity of the mutant receptor, whereas a specific PKA inhibitor antagonizes this increase. We propose that the hypersensitive ER $\alpha$  mutant breast cancer phenotype involves an integration of coupled acetylation and phosphorylation events by upstream signaling molecules.

## INTRODUCTION

Estrogen receptor (ER)  $\alpha$  is a member of a large family of nuclear steroid receptors that function as transcription factors mediating the mitogenic effects of estrogen. Because prolonged estrogen exposure is a known risk factor for developing invasive breast cancer (1), we hypothesized that the emergence of a mutated receptor could be an early event occurring in premalignant breast disease, and we subsequently identified a lysine to arginine somatic mutation at residue 303 (K303R) in ER $\alpha$  from human ductal hyperplasias that conferred increased responsiveness to subphysiologic levels of hormone (2).

The ligand-dependent activation function (AF) of ER $\alpha$  contained within the AF-2 domain and the ligand-independent AF-1 region contribute to the transcriptional activity of the receptor by recruiting coactivator proteins, such as the p160 and p300/CBP families (3–5). It is known that p300 enhances transcriptional activity through several different mechanisms, including bridging and scaffolding functions to join together other transcription factors with the basal transcriptional apparatus and histone acetylation (HAT) activity via acetylation of specific lysines contained within histone proteins (reviewed in McKenna *et al.*, ref. 5). In addition, p300 can directly acetylate nonhistone proteins, for example, the transcription factors p53, GATA-1, the ACTR coactivator, and the two steroid receptors androgen receptor (AR) and ER $\alpha$  (6–10). The complete consequences of nuclear recep-

tor acetylation are not yet appreciated, but acetylation of ACTR has been linked with transcriptional attenuation, whereas acetylation of AR has been linked with ligand-dependent activity (9–12).

It has been shown that phosphorylation and p300 acetylation/coactivator signals can be linked; for example, they coordinately regulate p53 activation of genes following DNA damage (13). However, it is not known whether phosphorylation and p300 acetylation events on ER $\alpha$  can occur coordinately. We do know that ER $\alpha$  is a frequent target for posttranslational phosphorylation modifications (14). ER $\alpha$  is phosphorylated and activated at several sites by multiple signaling kinases, including extracellular signal-regulated kinases 1/2 (15), p38 mitogen-activated protein kinase (MAPK; ref. 16), cyclin A/CDK2 (17), CDK-7 (18), c-Src (19–21), protein kinase A (PKA; refs. 22, 23), pp90-rsk1 (24), and AKT (25). These phosphorylation events influence ER $\alpha$  biological activity and in many cases are themselves downstream of membrane growth factor receptor signaling networks. Thus, growth factor stimulation of ER activity is likely to involve phosphorylation of the receptor and also has been implicated in the multifaceted problem of clinical resistance to the antiestrogen tamoxifen (26).

An emerging concept is that altered growth factor signaling can modulate the hormonal responsiveness of wild-type ER $\alpha$  (27), which is leading to new hormonal treatment sequencing strategies (28). Furthermore, ER-positive MCF-7 breast cancer cells can gradually adjust to long-term estrogen deprivation, resulting in a phenotype that has been termed adaptive hypersensitivity (29), with concomitant up-regulation of growth factor receptors (30) and/or activation of downstream signaling molecules such as extracellular signal-regulated kinases 1/2 and AKT (30–32). These studies led us to question whether activation of specific signaling molecules also might be involved in the hypersensitive phenotype associated with the K303R ER $\alpha$  mutation.

One candidate is the cyclic AMP (cAMP) signaling pathway, where ER $\alpha$  can be stimulated in the absence of estrogen either with the cAMP analog 8-bromo-cAMP (8-Br-cAMP) or a combination of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and cholera toxin, which increases cAMP via a G protein-mediated signal transduction pathway (33). cAMP-dependent PKA is a ubiquitous serine/threonine kinase that regulates a variety of cellular activities, such as the hormonal regulation of cells (34), and frequently is overexpressed in clinical breast tumors (35). There are four potential sites for phosphorylation by PKA in ER $\alpha$  (serines S236, S305, S338, and S518; ref. 36), with S236 being the best characterized site regulating receptor dimerization and DNA binding (23). These serines are all within the cAMP-dependent protein kinase phosphorylation sequences XRRXSX or SKKXSX. Interestingly, it has been shown that mutation of the SKKXSX recognition sequence to SKRXSX increases the kinetics of PKA phosphorylation (37) and predicts that the K303R mutation in ER $\alpha$  may generate a more efficient substrate for PKA phosphorylation at S305, a hypothesis that was addressed in the study herein in the context of its effects on hormone sensitivity and acetylation status.

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We previously reported that site-directed, double mutations of the ER $\alpha$  lysines 302 and 303 enhanced ligand sensitivity and decreased the acetylation status of the receptor (10). This result suggested that acetylation modification itself might be a determinant for hormone sensitivity as originally described for the K303R ER $\alpha$  mutation (10). However, we now have prepared single mutations at K303 (nucleotide 908A) representing the other two possible nucleotide changes (A to T, K303 methionine, and A to C, K303 threonine) and found that although both of these two mutations abrogated ER acetylation, neither of them was hypersensitive to estrogen in ER transactivation assays. We conclude that hypoacetylation at the K303 acetylation motif does not fully explain the enhanced sensitivity associated with the K303R ER $\alpha$  mutation. However, we found that acetylation and phosphorylation of this region were inversely related and that specific phosphorylation by PKA blocked the acetylation of ER $\alpha$  and further enhanced the estrogen sensitivity of the mutant receptor. These results provide the first evidence that phosphorylation and acetylation of ER $\alpha$  are interrelated events and that phosphorylation at S305 antagonizes acetylation, suggesting a potential cascade of posttranslational modifications of the receptor at these motifs.

## MATERIALS AND METHODS

### Expression Vectors and Reporter Constructs

Wild-type (WT) and K303R ER cDNA inserts from pcDNA1 (2) were subcloned into the *EcoRI* site of the pcDNA3.1 selectable expression plasmid (Invitrogen, Carlsbad, CA), and a hemagglutinin (HA) tag was added to the amino-terminus of ER $\alpha$  (38). K303T, K303M, S305A, S305D, and S294A ER $\alpha$  constructs were generated using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) to generate the indicated point mutations in pcDNA3.1-HA-WT ER $\alpha$ . The primer sequences for construction of the point mutations were as follows, with the mutated nucleotides italicized: K303M, GATCAAACGCTCTAAGATGAACAGCCTGGCCTTG; K303T, GATCAAACGCTCTAAGACGAACAGCCTGGCCTTG; S305A, GCTCTAAGAA(G) GAACGACCTGGCCTTGTCCTGACG; S305D, GCTCTAAGAA(G)G AACGCCCTGGCCTTGTC CTGACG; and S294A, GCCAACCT TTGGCCGACCCCGCTCATGATCAAACG. The entire nucleotide sequence of the construct then was verified using standard DNA sequencing procedures. The bacterial expression vectors for glutathione *S*-transferase (GST) fusion proteins of the ER $\alpha$  hinge region (residues 253 to 310) were constructed by cloning PCR-amplified fragments derived from their respective mammalian vectors into the *BamHI* and *EcoRI* sites of pGEX4T-1 (Amersham, Piscataway, NJ). Purification of bacterially expressed GST fusion proteins and p300 HAT has been described previously (2, 10, 38). The mammalian expression vector for GST-fused ER $\alpha$  hinge region (residues 253 to 310) was constructed by inserting the appropriate PCR-amplified GST-fused ER $\alpha$  hinge fragment into the *HindIII* and *EcoRI* sites of pcDNA3.1. To generate the yellow fluorescent protein (YFP) ER $\alpha$ - and K303R ER $\alpha$ -tagged vectors, the pECFP vector (39) was obtained from Dr. M. Mancini (Baylor College of Medicine, Houston, TX), and the CFP cDNA in this vector was replaced by YFP through *AgeI* and asparagine 718 digestion to generate pYFP-WT ER $\alpha$ . The COOH-terminal fragment WT ER $\alpha$  then was replaced with a *SmaI* and *BamHI* fragment of pEF-Tracer-K303R ER $\alpha$  to generate pYFP-K303R ER $\alpha$  expression plasmid.

### Cell Culture and Chemicals

SAOS-2, MDA-MB-468, COS-7, and HeLa cells were obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells originally were obtained from Dr. Benita Katzenellebogen (University of Chicago, Urbana, IL) but have been maintained in our laboratory for >10 years (2). All of these cell lines were maintained in minimal essential medium (Invitrogen) supplemented with 5% fetal bovine serum (Summit Biotechnology, Fort Collins, CO), 200 units/mL penicillin, and 200  $\mu$ g/mL streptomycin. Cells were incubated at 37°C in 5% CO<sub>2</sub>. The C4-12-5 MCF-7 subline was routinely maintained in  $\alpha$ -minimal essential medium without phenol red, 5% charcoal/dextran-treated fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mmol/L glutamine,

50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin. MTT reagent and 17 $\beta$ -estradiol were purchased from Sigma (St. Louis, MO), and 8-Br-cAMP and H89 were obtained from Calbiochem (La Jolla, CA). GSH-Sepharose and protein A-Sepharose, <sup>14</sup>C-acetyl-CoA,  $\gamma$ -<sup>32</sup>P-ATP, and <sup>32</sup>P-P<sub>i</sub> were purchased from Amersham. All of the other chemicals were purchased from Sigma unless otherwise stated.

### Stable Transfection and Growth Assay

C4-12-5 MCF-7 cells were derived by culturing MCF-7 cells in the absence of estrogen for 9 months and have lost detectable expression of endogenous ER $\alpha$  (40). C4-12-5 cells were stably transfected with YFP-WT ER $\alpha$  or YFP-K303R ER $\alpha$  plasmids as described previously (2). Transfected clones were screened for expression of ER $\alpha$  using immunoblot analysis. Proliferation of the ER $\alpha$  transfectants was assessed using MTT reagent (Sigma) following the manufacturer's protocol. Cells were maintained in the absence of estradiol and then treated for 4 days with increasing amounts of estradiol (E<sub>2</sub>; 0, 10<sup>-11</sup>, and 10<sup>-9</sup> mol/L) in the absence or presence of the PKA inhibitor isoquinolinesulfonamide H89 (10  $\mu$ mol/L), as described in the figure legends. Cell growth experiments were performed twice in triplicate. The data are represented as the average fold induction relative to no E<sub>2</sub>  $\pm$  SE.

### Transactivation Assays

Cells were transfected with Fugene 6 reagent (Roche Clinical Laboratories, Indianapolis, IN) for transient transactivation assays following the manufacturer's protocol and as described previously (38). Briefly, 2 days before transfection, the culture media were switched to phenol red-free media plus 5% dextran-coated charcoal-stripped fetal bovine serum. Twenty-four hours before transfection, cells were seeded into 12-well plates (Corning Inc., Corning, NY) at ~40 to 60% confluence, and 2 hours before transfection, the cells were switched to fresh media. Each well was cotransfected with 1  $\mu$ g ERE<sub>2</sub>-tk-luciferase reporter plasmid, plus 1 ng of the respective ER $\alpha$  expression plasmid, and 100 ng of CMV- $\beta$ -galactosidase expression plasmid as an internal control. Twenty-four hours after transfection, cells were treated with increasing amounts of E<sub>2</sub> (10<sup>-12</sup> to 10<sup>-9</sup> mol/L), as described in the figure legends. Cells were harvested after 18 to 24 hours of treatment with hormone, and ERE luciferase activities were normalized by dividing by the  $\beta$ -galactosidase activity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average  $\pm$  SE and are representative of at least three independent experiments. In some experiments, the PKA activator 8-Br-cAMP (10 mmol/L) or the PKA inhibitor isoquinolinesulfonamide H89 (10  $\mu$ mol/L) was added at the same time as estrogen, and an ERE-TAT-luciferase reporter (ERE-E1b-luc provided by Dr. Carolyn Smith, Baylor College of Medicine, Houston, TX; ref. 41) was used in the transactivation assay. Equal expression of protein was determined using immunoblot or immunoprecipitation with anti-ER $\alpha$  antibodies as indicated.

### Acetylation Assays

**In vitro Assay.** The bacterial expression vector for GST-p300 HAT was obtained from Dr. Richard Eckner (Zurich, Switzerland). GST-pulldown experiments were performed as described previously (2). A standard HAT assay subsequently was performed on 10  $\mu$ g of purified GST-ER protein (42) with 200 ng purified acetyltransferase (GST-p300) and 30  $\mu$ L HAT buffer [50 mmol/L Tris HCl (pH 8.0), 10% (v/v) glycerol, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L sodium butyrate; refs. 43, 44] in the presence of 50 nCi of [<sup>14</sup>C]-acetyl-CoA for 30 minutes at 30°C. The reactions were stopped by the addition of SDS loading buffer and then were resolved on 4 to 12% SDS-PAGE gels. After Coomassie Blue staining and destaining, the acetylated ER $\alpha$  and the p300 autoacetylation bands were visualized by autoradiography.

**In vivo Assay.** MCF-7 cells were grown in phenol red-free media with or without 5% stripped fetal bovine serum for 2 days, washed twice with ice-cold PBS, and disrupted in cell lysis buffer [20 mmol/L Tris HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L B-glycerophosphate, 1 mmol/L sodium orthovanadate, and 10% glycerol plus 1:100 proteinase inhibitor mixture III; Calbiochem]; 100  $\mu$ mol/L 8-Br-cAMP were added in some experiments. The cell lysates were cleared by centrifugation at 16,100  $\times$  g for 10 minutes at 4°C; protein concentrations were determined using BCA protein assay (Pierce,

Rockford, IL) following the manufacturer's protocol; endogenous acetylated proteins were immunoprecipitated from 500  $\mu\text{g}$  of the lysate using an antibody specific for acetylated lysine (1:100 dilution; Cell Signaling Technology, Beverly, MA) and protein A-Sepharose. After five washes with cell lysis buffer, the immunoprecipitates were boiled in SDS loading buffer for 2 minutes and centrifuged, and 4 to 12% SDS-PAGE was used to resolve the immunoprecipitated proteins. Antibodies against ER $\alpha$  (6F-11, 1:100 dilution; Vector Labs Inc., Newcastle, United Kingdom) or Amplified in Breast (AIB)1 (1:100 dilution; BD Transduction Lab, Los Angeles, CA) were used for immunoblot analysis of the acetylated proteins.

**Total Kinase Assay.** To detect the ability of ER $\alpha$  to serve as a specific kinase substrate for total cellular kinase activity, 20  $\mu\text{g}$  of the respective GST-ER $\alpha$  hinge fragments were immobilized onto GSH-Sepharose beads, and after two initial washes in 500  $\mu\text{L}$  kinase buffer [25 mmol/L Tris HCl (pH 7.5), 5 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L sodium orthovanadate, and 10 mmol/L  $\text{MgCl}_2$ ], the beads were incubated with 200  $\mu\text{g}$  of MCF-7 cell lysate in rotation, as described previously, for 2 hours at 4°C. The beads then were washed three times with lysis buffer and two times in kinase buffer, and the beads were incubated with 10 nmol/L ATP in the presence of 0.25  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$ -ATP in 30  $\mu\text{L}$  kinase buffer for 20 minutes at 30°C. The reactions were stopped by the addition of SDS loading buffer and boiled for 2 minutes. After a 2-minute centrifugation, the reactions were resolved on 4 to 12% SDS-PAGE gels. After staining and destaining of the gel with Coomassie Blue dye, the gels were dried, and phosphorylated ER $\alpha$  bands were visualized by autoradiography.

**PKA Kinase Assay.** Twenty micrograms of GST-ER $\alpha$  hinge protein were incubated with 1.0 units of recombinant PKA catalytic subunit (Calbiochem) in the presence of 0.25  $\mu\text{Ci}$   $\gamma$ - $^{32}\text{P}$ -ATP and 10 nmol/L ATP in 30  $\mu\text{L}$  kinase buffer for 20 minutes at 30°C. The reactions were stopped by the addition of SDS loading buffer, and the reactions then were resolved on 4 to 12% SDS-PAGE gels. After staining and destaining of the gel with Coomassie Blue dye, the gel were dried, and phosphorylated ER $\alpha$  bands were visualized by autoradiography.

### *In vivo* Labeling of ER $\alpha$ Hinge Domain with $^{32}\text{P}$ -P $_i$

Mammalian expression vectors for GST-fused WT, K303R, and K303R/S305A ER $\alpha$  hinge regions (residues 253 to 310) were transiently transfected into COS-7 cells with Fugene 6 transfection reagent. Thirty-six hours later, cells were switched to phosphate-free Dulbecco's modified Eagle's media (Invitrogen) for 2 hours and then switched to the same media supplemented with 5% dialyzed fetal bovine serum with and without 330 nmol/L trichostatin A (TSA) for 4 hours. After the addition of 9 mCi  $^{32}\text{P}$ -P $_i$  with or without 100  $\mu\text{mol/L}$  8-Br-cAMP, the cells were metabolically labeled for another 4 hours at 37°C. After removal of the labeling media, cells were washed twice with ice-cold PBS, and 500  $\mu\text{L}$  hypertonic buffer [400 mmol/L NaCl, 20 mmol/L Tris (pH 7.6), 1 mmol/L EDTA, 1 mmol/L DTT, 50 mmol/L NaF, 200  $\mu\text{mol/L}$   $\text{Na}_3\text{VO}_4$ , 20 mmol/L glycerol (v/v), and 1:100 diluted proteinase inhibitor mixture III; Calbiochem] were added to each plate. The cells were lysed by three cycles of freeze (dry ice)/thaw (ice), and cell lysates were cleared by ultracentrifugation (105,000  $\times g$ ) for 20 minutes at 4°C. The cell lysates then were adjusted to contain 1% Triton X-100 and 1% Tween-20, and 20  $\mu\text{L}$  50% GSH-Sepharose slurry were added and incubated at 4°C for 30 minutes in rotation. After washing five times with hypertonic buffer containing 1% Triton X-100 and 1% Tween-20, the beads were boiled in SDS loading buffer and collected by centrifugation. The supernatants were resolved onto 4 to 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. The phosphorylated ER $\alpha$  bands then were visualized by autoradiography, and the GST-ER $\alpha$  protein levels were determined by immunoblot of the same membrane with an anti-ER $\alpha$  antibody (SPA-100, 1:400 dilution; StressGene, Victoria, BC, Canada), which is specific for a region (residues 281 to 300) within the ER $\alpha$  hinge domain.

### PKA Kinase Coupled Acetylation Assays

Different GST-ER $\alpha$  hinge fragments were phosphorylated by recombinant PKA catalytic subunit as we described previously. The kinase reactions then were stopped by the addition of 10 mmol/L EDTA in 500  $\mu\text{L}$  PBS containing 1% Triton X-100 and 1% Tween-20, followed by the addition of 20  $\mu\text{L}$  50% GSH-Sepharose slurry. After a 30-minute incubation at 4°C in rotation, the

beads were washed three times with PBS containing 1% Triton X-100 and 1% Tween-20 and then two times with the HAT assay buffer. The acetylation reactions then were initiated by adding 200 ng purified acetyltransferase (GST-p300) and 500  $\mu\text{mol/L}$  acetyl-CoA in 30  $\mu\text{L}$  HAT buffer for 30 minutes at 30°C. The reactions were stopped by the addition of SDS loading buffer, and the GST-ER $\alpha$  fragments were eluted by boiling. After centrifugation, the supernatants were resolved onto 4 to 12% SDS PAGE gels and transferred onto nitrocellulose membranes. The acetylated proteins were visualized by immunoblot analysis with an antiacetylated lysine antibody (Cell Signaling; 1:1000 dilution), and the phosphorylated proteins were visualized by autoradiography of the same gel. The total protein levels of the GST-ER $\alpha$  fragments were determined by Ponceau S (Sigma) staining.

### Immunoblot Analysis

After blocking of the transferred nitrocellulose membranes with Tris-buffered saline/Tween [20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween-20] supplemented with 5% nonfat milk for 1 hour at room temperature, the membranes were incubated with primary antibodies either for 1 hour at room temperature (anti-ER $\alpha$  and anti-AIB1) or overnight at 4°C (antiacetylated lysine and anti-GST), incubated with secondary antibodies for 1 hour at room temperature, and developed with enhanced chemiluminescence reagents (Amersham). Anti-ER $\alpha$  (6F11; Novacastra, Newcastle, United Kingdom) and anti-AIB1 antibodies were used at 1:100 dilution; anti-ER $\alpha$  (SPA-100) was used at 1:400 dilution; anti-HA11 (Novacastra) was used at 1:100; and anti-Rho-GDI (A-20; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:200 dilution.

## RESULTS

**Hypoacetylation of K303 ER $\alpha$  Does Not Explain the Hypersensitive Phenotype.** Previously we generated double mutations of K302/303 and examined their effect on estrogen response (10). However, because an A to G transition (K303R) at ER $\alpha$  K303 was the only change ever found in premalignant breast lesions (2), we hypothesized that this specific residue alteration might be biologically relevant and provide an altered hormone response to cells. To test this hypothesis, we generated the two other possible single nucleotide substitutions (A to T or C, K303M or K303T) at the K303 residue and tested their transcriptional activity in estrogen-induced ERE-luciferase reporter assays (Fig. 1A). We transiently transfected HeLa cells with a WT ER $\alpha$  expression vector and expression vectors for K303R, K303M, or K303T and then determined ER $\alpha$  activity using increasing amounts of estrogen. Transcriptional activity of the K303R mutant receptor was induced even at the lowest concentration of added estradiol ( $10^{-12}$  mol/L), and activity was higher at  $10^{-9}$  mol/L estradiol than with WT receptor. However, transactivation by K303M and K303T was similar to WT ER $\alpha$ . Similar levels of expression of ER $\alpha$  protein were seen with these mutant expression plasmids (Fig. 1B). These results suggest that only the specific K303R substitution results in a receptor with enhanced sensitivity to estradiol.

Acetylation of ER $\alpha$  by p300 is preferentially at residues K302 and K303 within the receptor hinge domain, with K303 being the major acetylation site (10). To test the consequences of K303 mutations on protein acetylation, *in vitro* acetylation assays were performed using recombinant p300 and GST fusions of the various mutant receptors (ER $\alpha$  residues 253 to 310) were used as an acetylation substrate to include the potential S305 PKA site to be studied next and to exclude the other potential PKA sites contained within ER $\alpha$ , and the efficiency of incorporation of [ $^{14}\text{C}$ ]acetyl-CoA was assessed after electrophoresis of the reactions on an SDS polyacrylamide gel (Fig. 1C, top). As expected, autoacetylation of GST-p300 was seen in all of the lanes, and WT ER $\alpha$  was efficiently and selectively acetylated by p300. In contrast, all of the K303 substitutions were hypoacetylated. We determined that equal amounts of proteins were analyzed in the

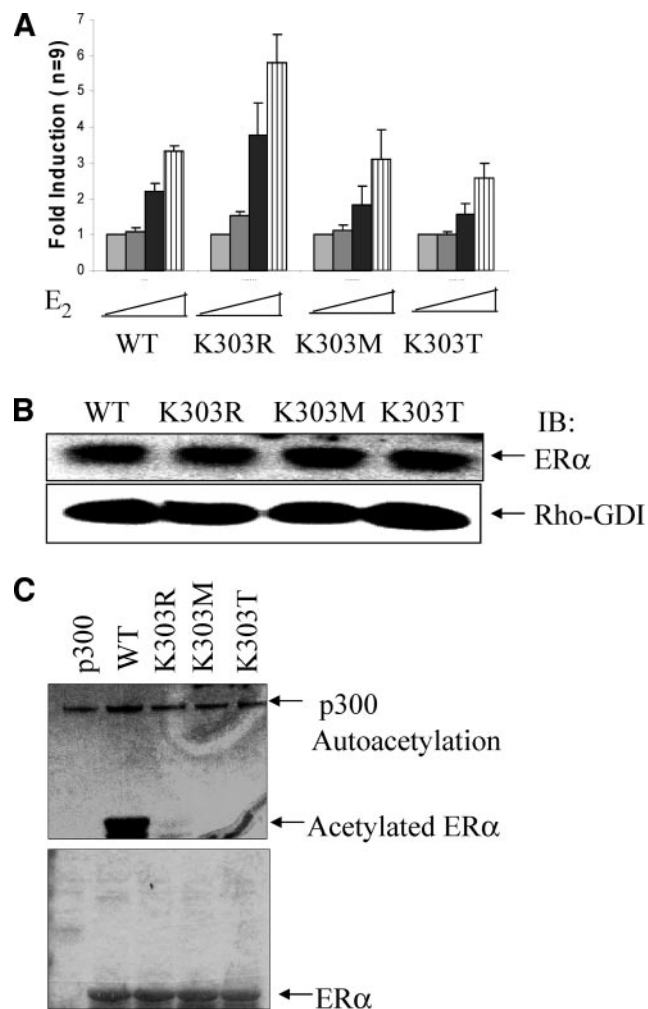


Fig. 1. Hypoacetylation does not fully explain estrogen hypersensitivity. *A*, HeLa cells were transiently transfected with vectors expressing WT ER $\alpha$  or the various K303 site mutants, and luciferase activity was measured in the absence or presence of  $10^{-12}$ ,  $10^{-11}$ , and  $10^{-9}$  mol/L E $_2$ . Fold induction was calculated on nine independent experimental assays performed in triplicate. *B*, immunoblot analysis of extracts from *A* with anti-ER $\alpha$  and Rho-GDI antibodies. *C*, *In vitro* acetylation assays were performed on GST-ER $\alpha$  fragments, and acetylated proteins were visualized by autoradiography (*top*), whereas input protein was visualized using Coomassie Blue staining of the gel (*bottom*).

acetylation assays by Coomassie Blue staining of the GST protein fragments (Fig. 1C, *bottom*). We conclude that a failure in acetylation of ER $\alpha$  does not fully explain the hypersensitive transcriptional phenotype of the K303R mutation.

**ER $\alpha$  Protein Acetylation and Phosphorylation Are Inversely Correlated.** Current models advocate that growth factor stimulation of ER activity is likely to be mediated through phosphorylation of ER $\alpha$  itself (45) and that phosphorylation of ER $\alpha$  in MCF-7 cells in culture can be accomplished via the addition of growth factors present in serum (46, 47). Therefore, we incubated MCF-7 cells in either medium stripped and devoid of growth factors and estrogen (Fig. 2, -) or in medium containing serum (Fig. 2, +) for 48 hours before preparation of cell lysates. The cell lysates then were divided and used for either a total *in vitro* kinase phosphorylation assay with GST-WT ER $\alpha$  as the substrate (Fig. 2A) or for an immunoprecipitation and immunoblot analysis (Fig. 2B). As expected, total ER $\alpha$  phosphorylation was increased with the addition of serum to the media (compare the - and + lanes, Fig. 2A). Concurrent with this increase in ER $\alpha$  phosphorylation, we found that the levels of acetylated ER $\alpha$  were decreased (Fig. 2B; immunoblot/ER $\alpha$ ). As a control, we included AIB, which also has been shown to be acetylated by p300 (11).

However, we did not observe an inverse correlation between serum addition and protein acetylation of AIB. These results showing an inverse relationship between ER $\alpha$  acetylation and phosphorylation with serum suggested that these two processes might be coupled.

We next examined whether mutations in the acetylation motif (K303R, K303M, or K303T) would affect the ability of ER $\alpha$  to serve as a substrate for *in vitro* kinase activity. To accomplish this, GST-fusion proteins of the receptors encompassing ER $\alpha$  residues 253 to 310 were used as substrates with a cellular extract of serum-stimulated MCF-7 cells (Fig. 2C and D). We found that the specific K303R ER $\alpha$  mutation, but not the other two mutations K303M or T, was an efficient phosphorylation substrate and furthermore that the K303R mutation might be a more efficient substrate than WT ER $\alpha$  for total kinase activity contained in MCF-7 cells (Fig. 2C). To address the location of the potential phosphorylation site within the GST-ER $\alpha$  fragments used as substrates, we mutated a conserved serine at residue 294 to alanine (S294A) to inactivate this site and mutated the serine at residue 305 to aspartic acid (S305D) to block phosphorylation; these GST fragments then were used in *in vitro* kinase assays (Fig. 2D). We found that the S294A mutation had little effect on ER $\alpha$  phosphorylation but that the S305D mutation greatly reduced the phosphorylation of the ER $\alpha$  fragment. This is consistent with previous findings that the S294 site is not a major hormone-stimulated ER $\alpha$  phosphorylation site (48). This result led us to question next which specific cellular kinase might be responsible for phosphorylation of ER $\alpha$  S305.

**Phosphorylation of ER $\alpha$  S305 by PKA Blocks Acetylation of K303.** The consensus substrate sites for PKA are XRRXSX and XXXXSX, and it is known that a number of important transcriptional activators, such as CREB protein and APAF-1, and the protein phosphatases CDC25 and protein phosphatase 1 (PP1) contain consensus PKA sites with either a K or an R at the -2 or -3 positions (Fig. 3A; refs. 49–52). The potential PKA site in WT ER $\alpha$  S305 (SKKNSL) has been noted previously (48), but it has not been characterized as a direct substrate for PKA phosphorylation (36). The K303R ER $\alpha$  mutation retains this potential PKA consensus site; therefore, we first tested whether it could be directly phosphorylated by recombinant PKA in an *in vitro* kinase assay using GST-ER $\alpha$  fusion fragments (Fig. 3B). Using equal amounts of the various ER constructs as substrates (Fig. 3B, *bottom input panel*), we found that the K303R ER $\alpha$  mutant was the most efficiently phosphorylated fragment compared with either WT or the K303M/L and T mutants (Fig. 3B, *top*). There was no endogenous, residual kinase in these reactions, as evidenced by our inability to detect phosphorylated ER $\alpha$  protein in the -PKA reactions (Fig. 3B, *middle*).

To confirm the S305 site as an authentic *in vivo* ER $\alpha$  phosphorylation site and whether this site could be affected by acetylation, we transfected COS-7 cells with the GST-WT, K303R, and K303R/S305A ERs, treated the cells with 8-Br-cAMP to activate PKA signaling, or 8-Br-cAMP plus the selective histone deacetylase inhibitor TSA, and then metabolically labeled the cells with radioactive organophosphate. The cells were harvested; the fragments were collected on GSH-Sepharose; and the eluates were run on SDS-PAGE (Fig. 3C). Autoradiography of the membranes revealed that WT ER $\alpha$  was faintly phosphorylated *in vivo* with 8-Br-cAMP treatment, which could be blocked by TSA cotreatment. The K303R ER $\alpha$  mutant was efficiently labeled *in vivo* after activation of PKA (Fig. 3C, *top*). In contrast to WT receptor, however, TSA was unable to block phosphorylation of the K303R mutant because acetylation at K303 is absent in this mutant. The phosphorylation status of the K303R/S305A double-mutant ER $\alpha$  protein was unaffected by PKA activation or TSA. The membranes then were probed with an anti-ER $\alpha$  hinge-specific antibody to visualize the input levels of the different ER $\alpha$  fragments (Fig.

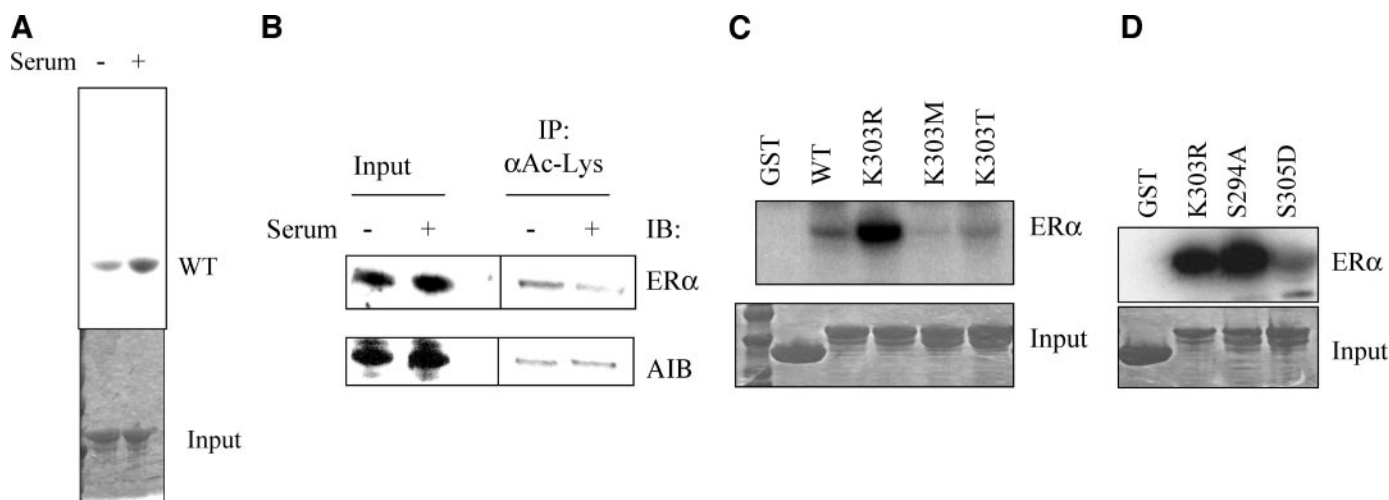


Fig. 2. Acetylation and phosphorylation are inversely related. *A*. MCF-7 cells were treated with (+) or without (–) serum addition, and total cellular extracts were used to perform an *in vitro* kinase assay of GST-WT ER $\alpha$  as substrate (*top*). The levels of input ER $\alpha$  protein were visualized using Coomassie Blue staining of the gel (*bottom*). *B*. Extracts from *A* were immunoprecipitated with antiacetylated lysine antibody, and the immunoprecipitates subjected to immunoblot (*IB*) analysis with antibodies to ER $\alpha$  (*top*) or AIB1 (*bottom*). The amount of protein used in the immunoprecipitation reactions is shown as input. *In vitro* kinase reactions using indicated GST-ER $\alpha$  fragments as substrate and MCF-7 serum-treated extracts to provide kinase activity appear in *C* and *D*, with autoradiography shown in the top panels and the level of input ER $\alpha$  protein revealed by Coomassie Blue staining shown below.

3C, *bottom*). This result suggests that phosphorylation and acetylation are uncoupled in the K303R ER $\alpha$  mutant.

To examine phosphorylation and protein acetylation simultaneously, an *in vitro* prephosphorylation reaction was first carried out using recombinant PKA catalytic subunit, and an *in vitro* acetylation reaction then was undertaken with different GST-ER $\alpha$  fragments (Fig. 3D). The level of acetylated ER $\alpha$  protein then was monitored using an antiserum to acetylated lysine (Fig. 3D, *top*). In the absence of PKA, the WT and the S305A ER $\alpha$  fragments were acetylated. As previously shown in Fig. 1, the K303R mutant was hypoacetylated. In the presence of PKA, the acetylation of WT ER $\alpha$  was blocked. The acetylation levels of the S305A ER $\alpha$  mutant were unchanged by PKA as expected. The levels of phosphorylated ER $\alpha$  proteins were visualized by autoradiography of the membrane (Fig. 3D, *middle*), and we found that the phosphorylation of the K303R mutant was enhanced compared with WT ER $\alpha$ . Finally, the input in these reactions is shown in Fig. 3D, *bottom*; equivalent levels of GST-ER $\alpha$  fragments were examined. To confirm this observation, we next treated MCF-7 cells with 8-Br-cAMP and examined the *in vivo* acetylation status of endogenous ER $\alpha$  and AIB using immunoprecipitation with an antibody to acetylated lysine (Fig. 3E). Treatment with the PKA activator reduced the levels of acetylated endogenous ER $\alpha$  protein, whereas the acetylation status of AIB was unaffected. These collective results show that the S305 site within the ER $\alpha$  hinge domain is an authentic site for cAMP-induced phosphorylation *in vivo* and furthermore that its phosphorylation can antagonize acetylation at the K303-centered motif.

**Modulation of PKA Intracellular Signaling Influences the Hormone Sensitivity of ER $\alpha$ .** Because there are reported cell-type and promoter-specific differences in cellular responses to PKA activators (33, 36, 53), we used HeLa cells transiently transfected with either WT or the K303R mutant receptor. A simple promoter reporter, ERE-TATA-luciferase, was used to examine the transcriptional activity of the receptors in the presence of estrogen, with either a PKA activator (PKA Act) or inhibitor (Fig. 4; H89). WT receptor exhibited a similar magnitude of response to estrogen, regardless of the addition of the PKA activator 8-Br-cAMP; in contrast, the transcriptional activity of the mutant receptor was enhanced at all of the concentrations of hormone tested with the addition of 8-Br-cAMP (Fig. 4A;

KR + PKA Act). Treatment with H89, a PKA-selective inhibitor, decreased estrogen-induced activity of the mutant and WT receptors (Fig. 4B). Thus, using this reporter and cell system, we were able to show differences in the net effect of PKA signaling on the transcriptional activity of the mutant receptor.

To examine whether H89 could inhibit the hypersensitive proliferative phenotype of K303R ER $\alpha$ -expressing breast cancer cells, we stably transfected WT ER $\alpha$  or the K303R mutant into an MCF-7 subline devoid of ER, so that the effects of the inhibitor could be separately analyzed in WT or K303R-only expressing cells. The effect of estrogen in the absence or presence of the PKA inhibitor on proliferation was examined (Fig. 4C and D), and the levels of exogenous ER $\alpha$  are shown in Fig. 4E. The K303R ER $\alpha$  stable transfectants (clones kr1 and 2) exhibited increased growth responses to low levels of estradiol ( $10^{-11}$  mol/L) compared with WT ER $\alpha$ -transfected cells (Fig. 4C, clones wt1 and 2;  $P < 0.001$ ). Thus, the hypersensitive proliferative phenotype we originally observed in MCF-7 cells coexpressing WT and the K303R ER $\alpha$  mutant (2) was recapitulated in these clones. H89 treatment blocked the hypersensitive phenotype of K303R (Fig. 4D) but had no effect on the estrogen-stimulated growth of WT ER $\alpha$ -expressing cells. Thus, inhibition of PKA signaling reversed the hypersensitive proliferation of mutant-expressing breast cancer cells. The next question we addressed was how mutation of the S305 PKA phosphorylation site would affect hormone sensitivity.

**Phosphorylation of S305 Mimics the Estrogen Hypersensitivity and Hypoacetylation Phenotype of the K303R ER $\alpha$  Mutant.** We compared the estrogen dose response of the phosphorylation mimic S305D mutant in transient transactivation assays in a number of different cell lines—human osteosarcoma SAOS-2 (Fig. 5A), HeLa (Fig. 5B), and human breast cancer MDA-MB-468 cells (Fig. 5C and D)—and found that the S305D ER $\alpha$  mutant exhibited greater estrogen sensitivity in all of these cells, suggesting that phosphorylation at this site is one determinant of estrogen hypersensitivity. This increase in sensitivity was not simply because of increased levels of the S305D protein in the transfected cells because equivalent levels of protein were detected in all of the transient transfection experiments as shown by either immunoblot (Fig. 5A and B, *bottom*) or immunoprecipitation and immunoblot analysis (Fig. 5C and D, *bottom*) for ER $\alpha$  and/or Rho-GDI as a control. When S305 was mutated to the nonphospho-

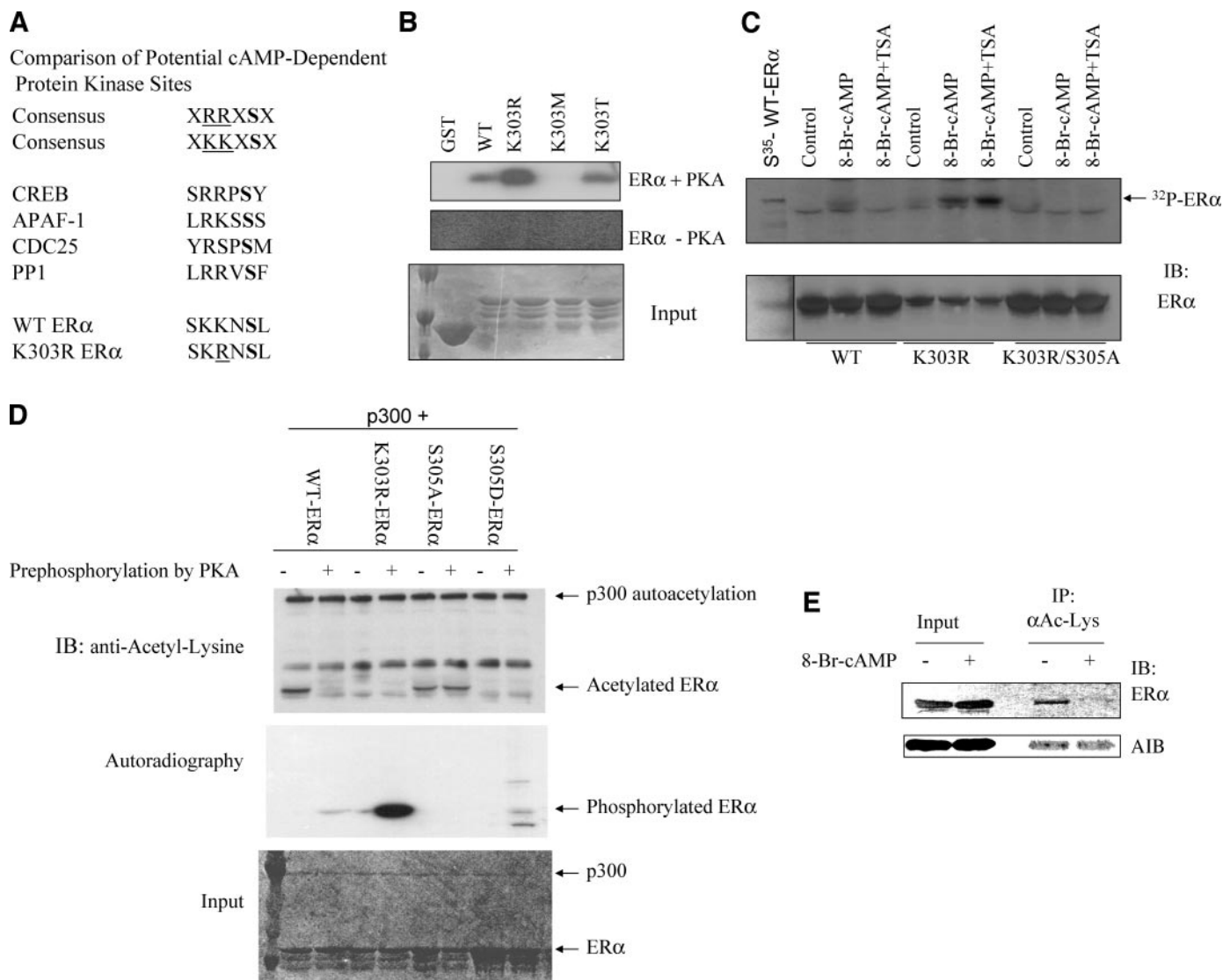


Fig. 3. PKA phosphorylation at S305 blocks acetylation of K303. **A**, schematic comparison of potential cAMP-dependent protein kinase sites in known PKA-inducible proteins along with two consensus PKA sites and the potential sites in ER $\alpha$ . **B**, *in vitro* kinase reactions containing various GST-ER $\alpha$  fragments used as substrate and recombinant PKA catalytic subunit as enzyme. Reactions were performed in the presence (ER $\alpha$  + PKA, *top*) or absence (ER $\alpha$  - PKA, *middle*) of PKA enzyme; the input GST proteins are shown in the bottom. **C**, COS-7 cells were transfected with the indicated GST-ER $\alpha$  hinge fragments, and cells were either left untreated (*Control*), treated with 8-Br-cAMP to activate PKA signaling, or 8-Br-cAMP plus TSA. The top panel shows autoradiography of the SDS-PAGE, and the bottom panel shows immunoblot (IB) analysis with anti-ER $\alpha$  antisera as a control for expressed protein. **D**, A PKA kinase-coupled acetylation assay was performed using recombinant PKA for prephosphorylation, followed by the addition of p300 for an *in vitro* acetylation assay. The top panel shows an immunoblot analysis of the reactions with an antiacetylated lysine antibody. p300 autoacetylation and acetylated (Ac) ER $\alpha$  bands are denoted with arrows. Autoradiography is shown in the middle panel, and the phosphorylated ER $\alpha$  bands are denoted with an arrow. Input ER $\alpha$  and p300 GST fragment proteins are shown in the bottom panel. **E**, MCF-7 cells were treated with (+) or without (-) 8-Br-cAMP, and total cellular extracts were immunoprecipitated (IP) with antiacetylated lysine antibody ( $\alpha$ Ac-Lys) and used for IB with anti-ER $\alpha$  or AIB.

rylatable alanine residue (S305A), it exhibited a similar activity compared with WT receptor; however, when we examined the activity of the double K303R/S305A mutant, we found that mutation of S305 abrogated the hypersensitivity of the K303R ER $\alpha$  mutant in breast cancer cells (Fig. 5A and D).

When we determined the acetylation status of these different single and double mutants at K303 and S305 ER $\alpha$ , we discovered that phosphorylation of S305 was firmly correlated with the acetylation status of the hinge domain acetylation motif (Fig. 5E). As expected, the K303R was hypoacetylated compared with WT receptor, and the single S305A and the two double K303R/S305A and K303R/S305D mutations had little effect on acetylation. In contrast, the phosphorylation mimic S305D ER $\alpha$  mutation exhibited significantly reduced acetylation *in vitro*. This result also was confirmed using immunoblots of the reactions shown in Fig. 5E with an antibody to acetylated lysine

(Fig. 5F). These combined results are consistent with our observations that phosphorylation and acetylation of ER $\alpha$  by PKA are inversely correlated and that a phosphorylation mimic at S305 (S305D) blocks acetylation of the receptor. We conclude that a coupled cascade of posttranslational ER $\alpha$  modifications may exist that is a key determinant of estrogen sensitivity and transcriptional response.

## DISCUSSION

Although the role of ER $\alpha$  phosphorylation is not fully understood, it does influence various aspects of transcriptional regulation, such as synergistic enhancement of estrogen-induced transcriptional activity (22), estrogen binding (54), interaction with coactivators (55), receptor stability (56), and nuclear localization (16). In this report, we show that the S305 site in ER $\alpha$  is a valid PKA *in vivo* phosphorylation site

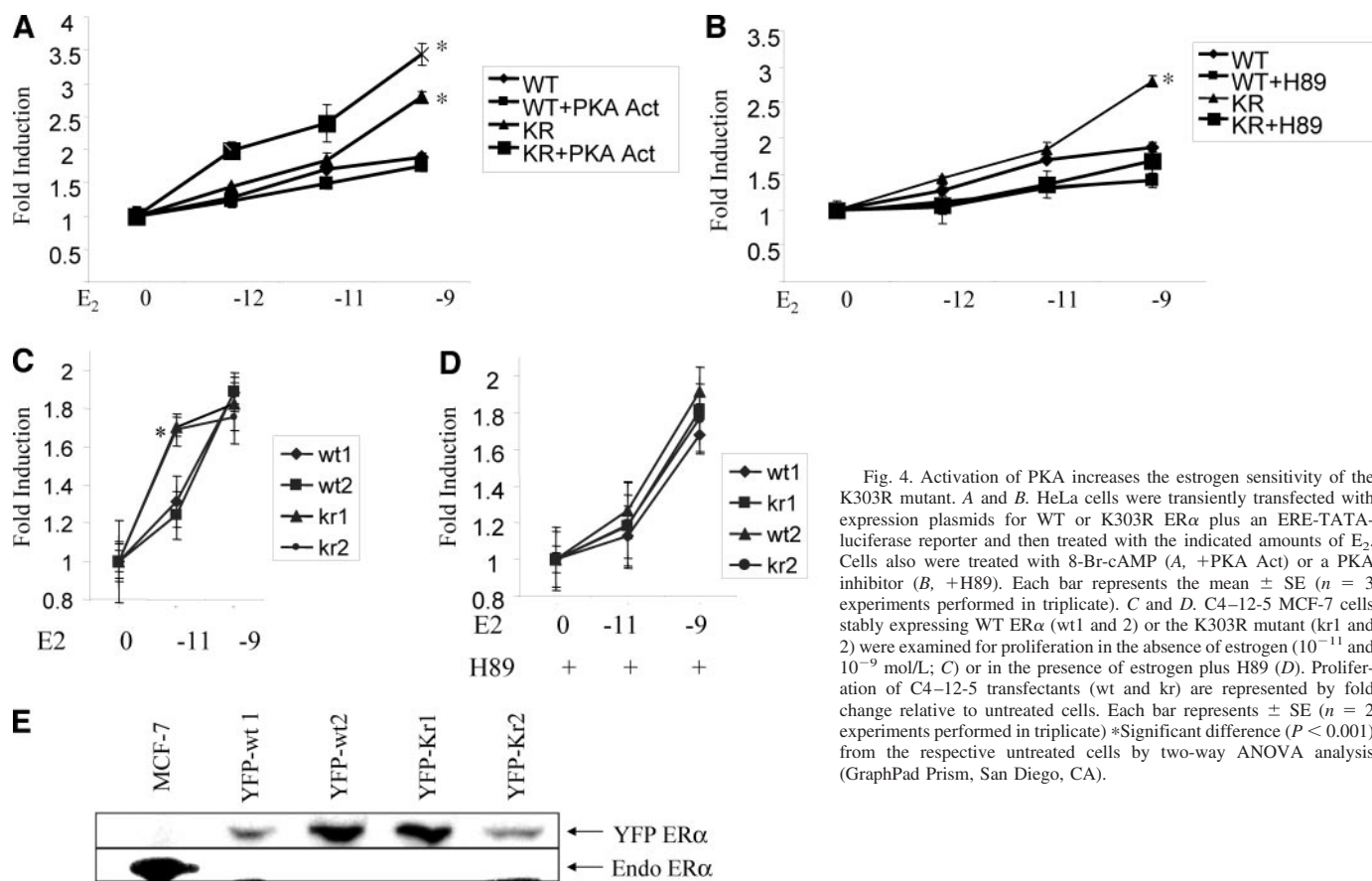


Fig. 4. Activation of PKA increases the estrogen sensitivity of the K303R mutant. *A* and *B*. HeLa cells were transiently transfected with expression plasmids for WT or K303R ER $\alpha$  plus an ERE-TATA-luciferase reporter and then treated with the indicated amounts of E<sub>2</sub>. Cells also were treated with 8-Br-cAMP (*A*, +PKA Act) or a PKA inhibitor (*B*, +H89). Each bar represents the mean  $\pm$  SE ( $n = 3$  experiments performed in triplicate). *C* and *D*. C4-12-5 MCF-7 cells stably expressing WT ER $\alpha$  (wt1 and 2) or the K303R mutant (kr1 and 2) were examined for proliferation in the absence of estrogen ( $10^{-11}$  and  $10^{-9}$  mol/L; *C*) or in the presence of estrogen plus H89 (*D*). Proliferation of C4-12-5 transfectants (wt and kr) are represented by fold change relative to untreated cells. Each bar represents  $\pm$  SE ( $n = 2$  experiments performed in triplicate). \*Significant difference ( $P < 0.001$ ) from the respective untreated cells by two-way ANOVA analysis (GraphPad Prism, San Diego, CA).

and furthermore that the naturally occurring K303R mutant is a more efficient substrate for phosphorylation by PKA. Although another group has examined the potential of the WT ER $\alpha$  S305 site to be activated via cAMP signaling, those experiments were conducted only in the context of a double mutation (S236A/S305A) in transactivation analyses with PKA activators and not in direct kinase assays (36). It also has been shown that the ER $\alpha$  S305 site can be an *in vivo* substrate for p21-activated kinase 1 (57). Given the variety of potential ER $\alpha$  phosphorylation events reported, it is notable that it may have been the increased efficiency of the K303R mutant site that made it possible to discern the apparent inverse relationship between phosphorylation and acetylation at the two overlapping K303/S305 acetylation and phosphorylation motifs in ER $\alpha$ .

We have reported previously the importance of the acetylation motif surrounding the K303 site in hormone-induced transactivation (10), and here we show that it is not acetylation status alone but associated phosphorylation of the adjacent K305 site that may be the key regulator of enhanced ligand sensitivity. Because enhanced ligand sensitivity of the phosphorylation mimic mutant of S305 was observed in a number of different cell lines and thus is not dependent on cellular context, it could suggest that the importance of phosphorylation and acetylation in this region of the receptor may be conserved. It is tempting to speculate that the exclusive isolation of the K303R ER $\alpha$  mutation in premalignant breast lesions (2) is because it is this sole nucleotide substitution that confers a potentially selectable phenotype (*e.g.*, increased substrate efficiency and ligand sensitivity).

There are other genes that can be regulated through an integration of site-specific phosphorylation and acetylation, but the mechanisms of how phosphorylation exerts effects on protein acetylation are largely unknown. A classical example is the covalent modification of histone tails, which is a critical step in regulating the transcription of

eukaryotic genes. Phosphorylation of histones, or of the HAT itself, may be one of the key regulators of histone protein acetylation. For example, phosphorylation of S10 of histone H3 is central for chromosome condensation and cell cycle progression, and S10 resides in close proximity to other residues that are modified via acetylation, K9 and K14, that impact on H3 function (58). It is interesting to note that K303 and S305 are similarly in close proximity in ER $\alpha$ .

Phosphorylation and protein acetylation can occur either in parallel or be inversely correlated. For example, casein kinase 2-dependent phosphorylation of the human positive coactivator 4 antagonizes p300-mediated protein acetylation (59), similar to what we report here for ER $\alpha$ . However, negative regulation of acetylation appears to be more unique because there are numerous examples in which phosphorylation of a substrate increases its acetylation. For example, phosphorylation of p53 tumor suppressor protein is directly correlated with its acetylation—the phosphorylation enhances acetylation, which consequently enhances p53's DNA binding ability and its repair activity (6, 60). Phosphorylation of p53 increases its ability to recruit CBP/p300 HAT, leading to an increase in its overall level of acetylation, suggesting a potential activation cascade (13). It recently has been shown that acetylation and phosphorylation of the AR are directly related and influence the effects of PKA and AKT signaling to the receptor (61). Whether a similar cascade of events occurs in the process of posttranslational modification of ER $\alpha$  and whether there are cellular mechanisms for coordination of ER $\alpha$  phosphorylation and acetylation remain to be determined.

Although it is known that ER $\alpha$  is a direct target for cAMP signaling, it has been shown that PKA also can target other proteins in steroid receptor complexes. ER coregulatory proteins, such as the SRC-1 coactivator (62) and CREB (36), can be phosphorylated via cAMP activation, but we do not completely understand the relative

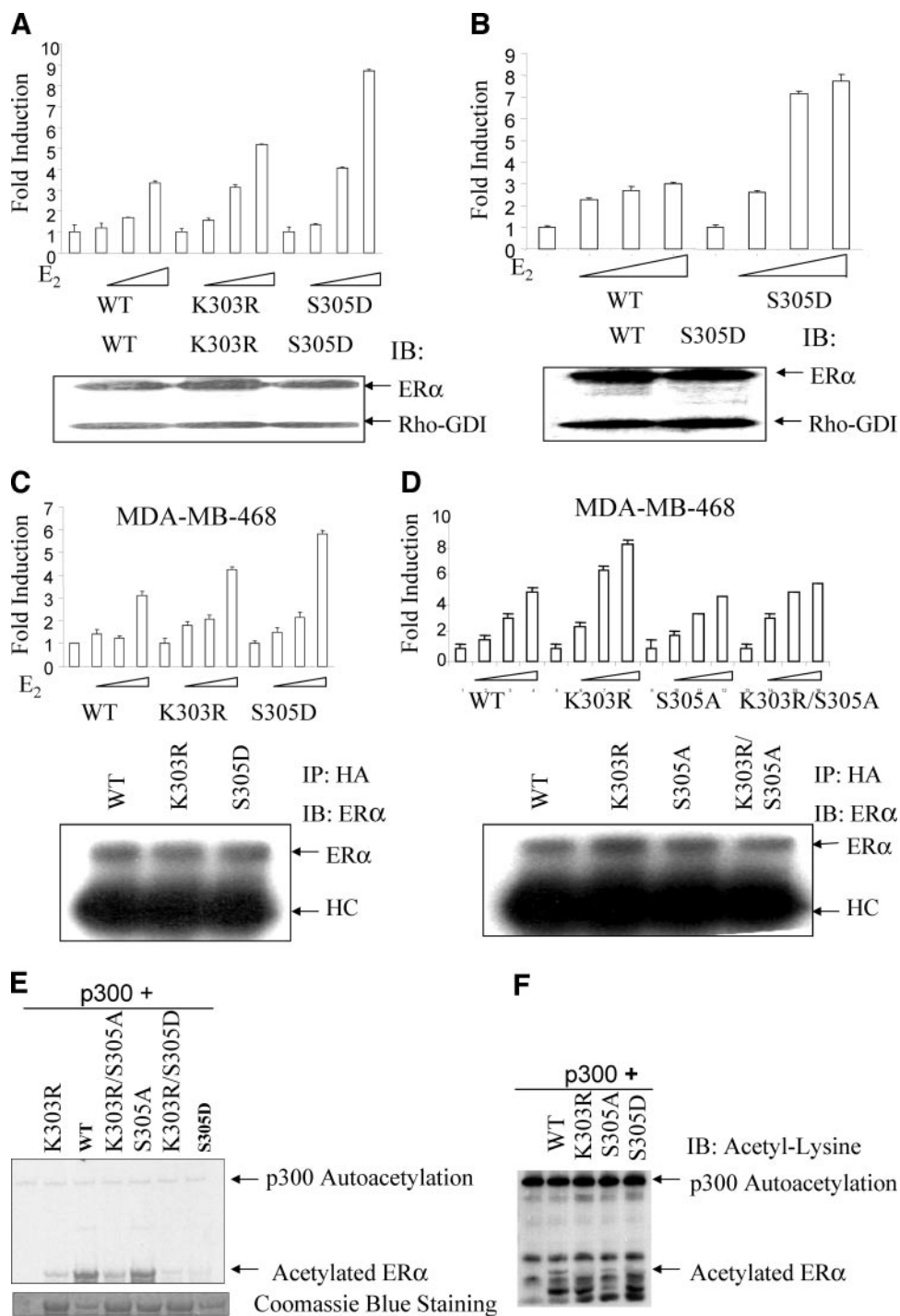


Fig. 5. Phosphorylation of ER $\alpha$  S305 modulates estrogen hypersensitivity of ER $\alpha$ . SAOS2 (A), HeLa (B), and MDA-MB-468 (C, D) cells were used in transactivation assays with the indicated ER $\alpha$  expression vectors with increasing amounts of  $E_2$  (0,  $10^{-12}$ ,  $10^{-11}$ , and  $10^{-9}$  mol/L). The bottom panels for A–D were immunoblotted for ER $\alpha$  (anti-6F11) and Rho-GDI (A, B) or immunoprecipitation with anti-HA11, followed by immunoblot (C, D) for ER $\alpha$ . E, an *in vitro* acetylation assay of WT, K303R, and double mutant GST-ER $\alpha$  fragments using p300 HAT. The p300 autoacetylation bands and acetylated ER $\alpha$  bands revealed by autoradiography are denoted with an arrow. F, an *in vitro* p300 acetylation assay in which the acetylated p300 and ER $\alpha$  bands were developed by immunoblot (IB) analysis with an acetylated lysine antibody. HC, IgG heavy chain

contribution of the phosphorylation of other proteins in the complex and their effects on ER transcriptional activity. We do know that the SRC-2 coactivator exhibits enhanced binding to the K303R ER $\alpha$  mutant receptor at low levels of hormone (2), but the role of phosphorylation by protein kinases such as PKA in this coactivator interaction is not presently known. The interaction between ER $\alpha$  and the cyclin D1 cell cycle regulatory protein can be modulated via cAMP second messengers (63), suggesting that an indirect alteration in the dynamic ER $\alpha$  transcription complex also could participate in enhanced responses.

It is becoming evident that alterations in the sensitivity of hormonal responses can occur in several ways. We have shown previously that

a gain-of-function mutation in the receptor itself (K303R ER $\alpha$ ) confers a hypersensitive growth response (2), and here we show that cAMP-dependent phosphorylation and signaling can further enhance this intrinsic sensitivity to hormone and that a PKA inhibitor can block the hypersensitive proliferation of these cells. Santen *et al.* (32) have shown that long-term deprivation of estrogen (termed adaptive hypersensitivity) leads to an activation of MAPK. However, in another estrogen-deprivation model of MCF-7 cells, it was shown that MAPK activation probably is not the sole pathway leading to the hypersensitive estrogen response (30). More recent data suggest that activation of phosphatidylinositol 3'-kinase and MAPK signaling may be involved in adaptive hypersensitivity (31). Furthermore, overex-

pression of CITED1, which binds CBP/p300 and ER $\alpha$ , also can enhance the sensitivity of MCF-7 cells to estrogen (64). The contributions of the ER $\alpha$  S305 site to estrogen response in these other models will have to be examined to determine whether this site also is a critical determinant of adaptive hypersensitivity. Michalides *et al.* (65) recently have shown that PKA signaling to ER $\alpha$  S305 also induces resistance to the antiestrogen tamoxifen. Interestingly, these authors showed that activation of PKA with an endogenous regulator, PKA-R1 $\alpha$ , also converts tamoxifen into an agonist, providing a potential mechanism for tamoxifen resistance in clinical samples. However, we have not observed that overexpression of the K303R ER $\alpha$  mutant confers resistance to tamoxifen in our transfected cell lines (ref. 2 and data not shown).

There are a number of potential clinical implications of this study. First, if PKA is confirmed to contribute to the development of estrogen hypersensitivity or resistance, especially in premalignant breast lesions, it may represent a new cellular target, which could be integrated with antiestrogen strategies for chemoprevention of breast cancer. Second, PKA also can interact with and impact on the EGFR pathway (66), so that PKA inhibitors could be developed to interfere with altered growth factor receptor signaling and the hypersensitive ER $\alpha$  phenotype in patients. Activated PKA also might be able to exert differential effects on cells that harbor the K303R mutation, potentially providing a selective advantage to these cells. We will focus future studies on the hypothesis that because the K303R ER $\alpha$  mutation confers a hypersensitivity to estrogen growth stimulation, if tumors expressing the mutation arise, then they may eventually escape from growth inhibition with aromatase inhibitors. Given the success of the third-generation aromatase inhibitors in the clinic (67), it is imperative that we dissect the molecular basis of estrogen hypersensitivity and determine whether resistance to aromatase inhibitors may arise through this mechanism.

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