Published in final edited form as:

Breast Cancer Res Treat. 2010 July ; 122(2): 381–393. doi:10.1007/s10549-009-0580-1.

A Hypersensitive Estrogen Receptor α Mutation that Alters Dynamic Protein Interactions

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Abstract

Estrogen receptor α (ER α) is highly regulated through multiple mechanisms including; cell signaling, posttranslational modifications, and protein-protein interactions. We have previously identified a K303R ER α mutation within the hinge region of ER α . This mutation results in altered posttranslational regulation and increased *in vitro* growth in the presence of low estrogen concentrations. We sought to determine if cells expressing this mutant ER α would display hypersensitive tumor growth in *in vivo* athymic ovariectomized nude mice. MCF-7 cells, stably expressing the K303R ER α , formed tumors in nude mice faster than cells expressing wild-type ER α in the presence of low levels of estrogen. When estrogen was withdrawn, all tumors regressed but half of the K303R ER α -expressing tumors became estrogen independent and regrew. We evaluated potential mechanisms for the observed hypersensitivity. The mutant ER α did not demonstrate increased estrogen binding affinity, but did exhibit increased interactions with members of the SRC family of coactivators. The mutant ER α demonstrated increased levels and occupancy time on the pS2 promoter. In the presence of the K303R ER α , the SRC-3 and p300 coactivators also displayed increased levels and time on the pS2 promoter. The K303R ER α has, in part, lost critical negative regulation by the F domain. Collectively, these data demonstrate an important role for the K303R ER α mutation in hormonal regulation of tumor growth and estrogen-regulated promoter dynamics in human breast cancer.

Keywords

breast cancer; estrogen receptor; K303R ER α mutation

Introduction

Long-term estrogen deprivation of breast cancer cells has been shown to result in adaptive hypersensitivity to low concentrations of estrogen ^{1–3}. When compared with wild-type MCF-7 cells, MCF-7 breast cancer cells that have undergone adaptive hypersensitivity display an increased ability to grow in nude mice under low estrogen conditions and growth

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can be inhibited when estrogen concentrations are increased⁴. Mutations within the ER α have also been shown to lead to estrogen hypersensitivity *in vitro*^{5,6}, however these data have not been confirmed by *in vivo* tumor growth like the adaptive hypersensitivity studies.

The serine at position 305 of ER α has been the subject of much interest. It is phosphorylated by a number of kinases including p21/Cdc42/Rac1-activated kinase 1 (Pak-1) and protein kinase A (PKA). While these kinases both phosphorylate this same ER α residue, they do not result in identical responses. For instance, ER α phosphorylation by Pak-1 results in a concomitant increase in S118 phosphorylation⁷, whereas phosphorylation by PKA leads to a reduction in S118 phosphorylation⁸. Expression of a constitutively- activated Pak-1 resulted in increased basal and estrogen-induced ERE-luciferase transactivation activity⁷, and siRNA to Pak-1 significantly suppressed estrogen-induced transcriptional activity⁹. In contrast, activation of PKA with forskolin resulted in a slight elevation of basal ER α activity, but it blunted estrogen-induced activity⁸. Thus, while Pak-1 and PKA can both phosphorylate ER α the S305 residue, the resulting biologic effects may be dependent upon the specific kinase.

Mutational analysis of S305 has demonstrated that this residue plays an important role in the regulation of ER α transactivation functions. It has been shown that mutating this serine to glutamic acid results in elevated basal and estrogen-induced activity⁹. We have shown that the S305D mutation also leads to increased estrogen sensitivity¹⁰. Analysis of ER α target proteins have confirmed these results. Cells expressing the S305E mutation were found to express higher levels of cyclin D1 and Zinc finger-147 genes¹¹. Furthermore, the cyclin D1 promoter was found to have higher activity with S305E but not with a S305A mutation¹¹. Forskolin activation of PKA leads to increased phosphorylation of S305 and increased the recruitment of ER α to the pS2 and c-Myc promoters⁸. Thus, mutation of S305 to mimic the phosphorylation of serine results in a general increase in ER transactivation and estrogen sensitivity.

We have identified a K303R ER α mutation, and demonstrated that it is hypersensitive for *in vitro* growth to low levels of estrogen⁵. Both the K303 and S305 residues lie within the hinge domain of ER α and we have previously shown that phosphorylation of S305 can inhibit acetylation at the K303 residue¹⁰. Thus, these two residues probably work in concert to regulate ER α activity. While activation of PKA signaling by 8-bromo-cAMP did not increase wild-type ER α transactivation, it significantly increased transactivation by the K303R mutant ER α ¹⁰. Additionally, this increased transactivation could be blocked by the PKA inhibitor, H89¹⁰. Previous binding analyses of the K303R ER α mutant have demonstrated that more SRC-2 coactivator is bound to the mutant receptor when compared with the WT receptor⁵. Additionally, the receptor corepressor MTA-1 can suppress the activity of both wild-type and the mutant K303R ER α ¹² whereas MTA-2 is only able to suppress the activity of the wild-type receptor¹³. Collectively, these data suggest a role for S305 phosphorylation and altered coregulator protein interactions in the hypersensitive phenotype of the K303R ER α mutant.

Previous work has demonstrated that ER α cofactors cycle on and off of the promoter in a dynamic manner^{14–16}. Elegant work by Reid et al have shown that unliganded receptor

cycles on and off of the pS2 promoter with a 20–30 minute cycling time and when estrogen is added, the cycling time increased to 30–50 minutes¹⁴. Two additional studies have shown a slightly longer cycling interval of 75–90 minutes in both the absence and presence of estrogen^{15, 16}. Similar cycling dynamics were also shown for ER α on the cathepsin D promoter¹⁵. Of note, in the presence of the proteasome inhibitor MG132, unliganded ER α only demonstrated one on/off cycle and in the presence of estrogen, the “on” cycle time remained the same, but the “off” cycle time was extended to almost 60 minutes¹⁴. Additionally, it has been shown that the dynamics of AIB1 binding on the pS2 promoter paralleled those of ER α ^{15, 16}. Collectively, these data demonstrate that the cycling dynamics of the promoter occurs in a regulated and context-dependent manner.

Together, these data led us to study whether the K303R ER α mutation may alter posttranslational regulation of the receptor, thereby influencing ER α function. In this manuscript, we seek to determine the molecular mechanisms leading to the hypersensitive phenotype of the K303R mutant ER α using both *in vivo* and *in vitro* assays. Here we show that cells overexpressing the K303R ER α mutation form larger tumors, when compared with WT ER α expressing cells in the presence of low estrogen concentrations. Furthermore, the K303R mutant protein displayed increased binding to additional members of the SRC family of coactivators. The K303R ER α mutant resides on the estrogen-regulated pS2 promoter for longer periods, and does not “cycle-off” with the same dynamics as wild-type ER α . Additionally, we demonstrate a possible role for helix 13 within the F domain of ER α receptor in the hypersensitive phenotype. Taken together, our data suggest that altered posttranslational regulation of ER α leads to increased binding of coactivators and reduced binding of corepressors, thus contributing to the hypersensitive phenotype of the mutant K303R ER α .

Materials & methods

Cell culture

Breast cancer cells were maintained on plastic in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 0.1 mM nonessential amino acids, 2 mM L-Glutamine, 50 U/ml penicillin/streptomycin (Gibco), and at 37°C with 5% CO₂-95% air. Unless otherwise noted, cells were passaged and removed from flasks when 70–80% confluent. For cell passage, cells were rinsed with PBS and trypsinized in 0.05% trypsin and 0.02% EDTA for 2 minutes at 37°C. Trypsin activity was quenched with the addition of medium containing 10% FBS.

Plasmid constructs

The mammalian expression vector for the full-length YFP-tagged wild type receptor (WT-ER α) and a YFP-tagged K303R mutant receptor (K303R-ER α), as well as the bacterial expression vectors for GST-ER α and GST-K303R-ER α hinge and hormone binding-domain have been previously described¹⁰. The mammalian expression vectors for SRC-1¹⁷, TIF2/SRC-2¹⁸, AIB1/SRC-3-3¹⁹, (GST-TFIIIB²⁰, GST-TBP) were obtained from Dr. Tsai MJ of Baylor College of Medicine, Houston, Texas.

Animal care and necropsy

Animals were housed and maintained in accordance with the animal care regulations of Baylor College of Medicine. All of the animal experiments were approved by the Baylor College of Medicine Animal Care and Use Committee pursuant to NIH guidelines. Female ovariectomized athymic nude mice purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN), maintained under specific pathogen-free conditions, and used for experiments at 5–6 weeks of age. Animals were sacrificed and necropsied when tumor burden was 1 cm³ or when animals were moribund. All procedures were performed while the animals were anesthetized with isoflurane (Abbot Laboratories, North Chicago, IL).

Tumor cell implantation

Cells expressing the wild-type or mutant ER α 's are described elsewhere ²¹. Briefly MCF-7 cells were engineered to overexpress YFP-WT ER α and YFP-K303R ER α plasmids and demonstrate similar levels of ER α expression ¹⁰. Cells grown in T150 flasks were given fresh media free of eukaryotic antibiotics two days prior to injection of cells. Cells in the log phase of growth were rinsed once with PBS, removed from the flask with 5 ml of Versene (Gibco), and collected with 10 ml of growth media. Cells were collected by centrifuging for 7 minutes at 1200 RPMs. The media was removed and all of the cells were resuspended in a total of 50 mls of media, centrifuged again, and resuspended at a concentration of 5×10^{-7} cells/ml of serum-free media. 5×10^{-6} cells were then aliquoted to one eppendorf tube per injection site and placed on ice. All procedures then on were carried out under a surgical hood in the animal facility. Nude mice were anesthetized with isoflurane. The #4 and #5 mammary gland teats were identified and the ventral area was cleaned with sterile alcohol pads followed by iodine. A small midline incision was made between the #4 teats and the dermis was separated from the peritoneum. Using a one-half CC syringe with a 27-gauge needle, 100 μ l of matrigel was mixed with 5×10^{-6} cells in 100 μ l media. A total of 200 μ l was injected into the #4 gland near the teat. The incision was closed with a single wound clip. The animals are then placed on their left side, and the area dorsal to the shoulder was cleaned with alcohol and iodine. A 0.72 mg, 60 day slow release pellet (Innovative Research of America; Sarasota, FL) or estrogen tubing with approximately 80 or 300 pg/ml release ²² was placed under the skin, above the shoulder, and the wound was closed with a wound clip. The animal was removed from anesthesia, placed in a warm cage and monitored until mobile.

Western blot analysis of tumors

Tumors were removed from animals at necropsy and a portion of the tumor was flash frozen in liquid nitrogen. Tumors (50–100 mg) were ground into a fine powder using a mortar and pestle that had been precooled with liquid nitrogen. The sample was lysed in 100 μ l lysis buffer per 10 mg of tumor (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na₃VO₄, 10% glycerol, 1 μ M okadaic acid, and Protease inhibitor cocktail, Roche, Basel, Switzerland). Thirty μ g of whole cell lysate were heated in Laemmli's sample buffer for 5 minutes, separated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane (Whatman Inc, Sanford ME), and probed with the following antisera, as indicated; anti-phosphoErk1/2 (Cell

Signaling Technology; Danvers, MA), anti-Erk1/2 (Cell Signaling Technology), anti-Cyclin D1/2 (Upstate; Charlottesville, NC), and anti-Actin (Sigma; St. Louis, MO). Antisera were as diluted in TBST (Tris-Buffered Saline-0.1% Tween 20 (v/v)) with 5% (w/v) dried milk or 5% (w/v) BSA. Peroxidase-conjugated secondary antisera, goat anti-mouse anti-serum (1:3000) (Amersham Biosciences; Piscataway, NJ), and goat anti-rabbit anti-serum (1:3000) (Amersham Biosciences); were used to detect the respective primary antibodies, and immunoreactive proteins were visualized with ECL chemiluminescence technology (Alpha Innotech; San Leandro, CA). Autoradiographic protein levels were quantified in the linear range of the film by scanning the image using a Canon LicoScan scanner and analyzing with the Scion Image™ software program (Scion Corp.; Frederick, MD). Each sample measured was calculated as the ratio of the average area of the phosphorylated protein over the average area of the respective total protein levels.

Ligand binding assay

Recombinant ER α and the K303R ER α mutant were *in vitro* translated using the TNT-coupled Reticulocyte Lysate System (Promega; Madison, WI) and diluted to a total volume of 2 ml in TESH buffer (10 mM TRIS, 1.5 mM EDTA, 1 mM DTT; pH 7.5). Fifty μ l of this dilution were mixed with 50 μ l of different concentrations of [2,4,6,7-³H]-17 β -estradiol (3.7 TBq/mol) with or without a 200-fold excess of unlabelled 17 β -estradiol, and incubated at 2 °C for 2 h. Unbound steroids were removed with dextran-coated charcoal and counts per min were determined by liquid scintillation counting.

GST pull-down assay

GST pull-down assays were performed as described previously²³ using the plasmid constructs indicated in the figures. The GST-fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia). Recombinant proteins (as indicated) were translated *in vitro* in the presence of ³⁵S-methionine using the TNT-coupled Reticulocyte Lysate system (Promega), and allowed to bind to the beads loaded with fusion protein in the absence or presence of estradiol as indicated. Proteins were separated by SDS-PAGE and submitted to autoradiography.

Immunoprecipitation

Hela cells were transiently transfected with expression vectors for AIB1¹⁹ with equal amounts of pcDNA3.1-wt ER α ²⁴ or K303R ER α plasmids²⁴, 24 hours later the cells were rapidly starved with phenol-red free media for three times, each time for 30 minutes, and then the cells were treated with increasing concentrations of estradiol for 24 hours, and then cells were lysed and immunoprecipitated with anti-ER antibody (H184, Santa Cruz Biotech, Santa Cruz, CA), after resolved onto SDS-PAGE and immunoblotted by anti-AIB1 as well as anti-ER α .

Chromatin immunoprecipitation

MCF-7 cells stably expressing WT or mutant ER α were treated with estrogen for the indicated times. Following treatment, cells were cross-linked with 1% formaldehyde at 37°C for 10 min and collected into PBS containing protease inhibitors. After centrifugation, cell

pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1) and sonicated, followed by centrifugation. Supernatants were diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1). To the precleared lysate, antibodies against ER (cocktail of Ab-1, Ab-3, and Ab-10; Neomarkers, Fremont, CA), AIB-1 (N-19; Santa Cruz; Santa Cruz CA), or p300 (C-20; Santa Cruz) were added, and the reaction was incubated overnight, followed by an addition of 45 μ L of protein A/G-sepharose and a further incubation for 1 hr. Sepharose beads were then collected and washed sequentially in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1). Beads were then washed once with TE buffer and extracted with 100 μ L of 1% SDS-0.1 M NaHCO₃. Eluate was heated at 65°C overnight to reverse the formaldehyde cross-linking. DNA fragments were then purified from the eluate of ER α , AIB-1, and p300 immunoprecipitates using a DNA purification kit (QIAquick spin, QIAGEN) and the fragments spanning the ERE sequence of human pS2 promoter were amplified with the primer and protocol described in ¹⁵.

ER α F domain analysis

The DNA fragments encoding the different ER α regions from amino acid 251 to amino acid 557, 566, as well as 554 were generated with PCR using either pcDNA3.1-wt ER α or K303R ER α plasmids as templates, 5' Bam HI and 5' Asp718 sites were designed in the forward and reverse primers respectively, the PCR fragments were subcloned into pBind vector (Promega Biotech, Madison, WI) through BamHI and Asp718 sites. The U2OS osteosarcoma cells were maintained in phenol-red free MEM supplemented with 5% charcoal-stripped FBS (Hyclone, Logan, UT) for 2 days. One day before transfection, cells were plated in 2 ml media at 2.5×10^5 per well in 6-well plates, and then transfected using Eugene 6 reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Each well was transfected with 1 μ g pG5-Luc (Promega Biotech, Madison, WI), 100 ng pCMV- β -gal vector, 10 ng various (as indicated in the Figure) pBind-ER α with 100 ng empty vector or TIF2 expression plasmid as indicated. Cells were treated with estrogen (E2, 10^{-9} M), or vehicle for an additional 18–24 hours, then the cells were washed twice with PBS, harvested into 1X reporter lysis buffer (Promega), and luciferase values as well as β -gal activities were assayed as described ²⁵. pG5-luciferase activity was normalized by dividing by the β -gal activity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average $\pm$ SEM, and are representative of three independent experiments. Data is presented as fold induction.

Statistical analysis

Statistical differences among groups were analyzed with student's t-test, Mann-Whitney or Chi-square analyses were appropriate. Analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

Results

The K303R ER α mutation renders cells hypersensitive to estrogen *in vivo*

Previously we have shown that the presence of the K303R ER α mutant renders a cell hypersensitive to low concentrations of estrogen *in vitro*⁵. We also wanted to determine if lower estrogen concentrations would favor the growth of cells expressing the mutant receptor *in vivo* in a xenograft tumor model. MCF-7 cells engineered to express WT-ER α or K303R-ER α were injected into the #4 gland of nude mice in the presence of 300–400 pg/ml or 80–100 pg/ml of circulating estrogen. Tumors were measured twice a week and tumor growth was plotted (Figure 1A). The data demonstrate that the receptor-expressing cells exhibited different tumor growth rates at the two different estrogen concentrations. The lower concentration of estrogen supported a more rapid growth rate of mutant ER α -expressing tumors (K303R-ER α vs. WT-ER α tumors, $p=0.0466$, chi-square), whereas the same cells grew more slowly in the presence of the higher levels of estrogen ($p=0.0018$, chi-square). WT-ER α -expressing cells did not demonstrate a difference in tumor growth rates at either estrogen concentration ($p=0.4011$, chi-square). Thus, while WT-ER α cells had similar growth rates in the presence of the two different concentrations of estrogen, as predicted based on their *in vitro* hypersensitive growth⁵, K303R-ER α tumors grew more rapidly in the presence of lower estrogen concentrations.

Three representative tumors from each group were analyzed by Western blot analysis to determine levels of phosphorylated mitogen activated kinases, ERK1/2 (pMAPK) and cyclin D1 levels as markers of proliferation. Tumors were crushed on liquid nitrogen and lysed as previously described²⁶. Western blot analysis demonstrated similar levels of phospho-MAPK levels and cyclin D1 between the WT-ER α and K303R-ER α tumors and both estrogen concentrations (Figure 1B). This analysis also demonstrates that both estrogen concentrations led to similar levels of activated pMAPK and cyclin D1 proteins.

To further analyze the role of the mutant ER α *in vivo*, WT-ER α and K303R-ER α cells were injected into nude mice in the presence of high-dose (300–400 pg/ml) estrogen. When the tumors had reached 150–200 mm³, animals were randomized into two groups, to continue high-dose estrogen or switched to no estrogen treatment. WT and mutant-expressing tumors in the high dose groups continued to grow and all animals had to be sacrificed within 6 months of injection. Similar to our first experiment, there was no difference in tumor growth between WT and mutant ER α -expressing tumors under high E2 concentrations (data not shown). All tumors that were switched to the low dose of estrogen slowly regressed and shown in Figure 1C was the time to complete regression, i.e. no palpable tumor. The graph demonstrates that WT-ER α cells regressed with a median time to complete regression of 9 weeks. All WT-ER α expressing tumors ($n=5$) demonstrated complete tumor regression in the absence of physiologic estrogen. Furthermore, after 14 months of monitoring, no WT-ER α tumors developed estrogen independence that would result in tumor regrowth. While K303R-ER α tumors ($n=8$) also regressed, the median time to complete regression was longer (29 weeks $p=0.0185$, chi-square vs. WT-ER α expressing cells), and one of eight tumors did not regress beyond a palpable size. Additionally, after only 35 weeks, K303R-ER α tumors developed estrogen independence, and 4 of 8 tumors began to grow again

within 63 weeks of tumor cell injection. Thus, cells expressing the mutant ER α exhibited a much greater time for tumor regression following estrogen withdrawal, and 50% of the estrogen-withdrawn tumors developed estrogen-independence. Collectively, these data suggest that the K303R ER α mutation confers an estrogen-independent growth advantage to breast cancer. These results predict that estrogen ablation therapies may be less effective when tumors express the K303R-ER α mutant. Recently, we have confirmed that indeed the mutation confers resistance to the aromatase inhibitor anastrozole ³⁹.

Estrogen binds wild-type and mutant ER α with similar affinities

To determine if this hypersensitive increase in proliferation and transcriptionally-active ER α was due to altered receptor affinity for either estrogen or DNA, we first examined estrogen binding affinities using saturation ligand binding analysis of *in vitro* translated WT and mutant ER α proteins. In Figure 2 representative Scatchard plots of saturation ligand binding assays with [³H]-estradiol are shown. The Scatchard analysis yielded an average dissociation constant (K_d) of 0.56 nM for both wild-type (Figure 2A) and mutant (Figure 2B) ER α ; these K_d values are well within the range generally reported for estradiol binding to ER α in a number of different assays ²⁷. Therefore the hypersensitive phenotype of the K303R mutation is not the result of an obvious change in the affinity of the receptor for estradiol.

Altered coregulator interactions

To analyze the mechanisms of estrogen hypersensitivity of the K303R ER α mutant, we examined its interactions with various coregulator proteins known to affect ER α transcription. Previously we have shown an increased interaction between the mutant ER α and SRC-2 coactivator ⁵. Therefore we next analyzed the protein-protein interactions of WT and mutant ER α with two other SRC coactivator family members using *in vitro* glutathione-S-transferase (GST) pull-down assays ²³. GST-WT-ER α and GST-K303R-ER α fusion constructs containing the hinge and hormone-binding domains were prepared. SRC family members were synthesized *in vitro* in the presence of [³⁵S] methionine and then tested for specific hormone-dependent binding to the immobilized GST-ER fusion proteins. Confirming our previous results ⁵, SRC-2 bound both receptors in the presence estradiol (10^{-6} M), but not in the absence of estradiol (data not shown). AIB1 also demonstrated increased binding to the mutant ER α at lower concentrations of estrogen when compared with WT ER α (Figure 3A). The binding of AIB1 and ER α was confirmed by immunoprecipitation (IP) of AIB1 from HeLa cells transiently transfected with WT or mutant ER α , followed by immunoblot analysis for ER α . Figure 3B demonstrates that mutant ER α exhibited increased binding to AIB1 *in vivo* in the absence (compare lanes 1 and 4), and the presence of 10^{-11} and 10^{-9} M estrogen (compare lanes 2 and 3 with lanes 5 and 6). Quantitation of this data is shown in Figure 3C. Collectively, these data demonstrate that the K303R ER α mutant receptor binds the SRC 2 and AIB1 coactivators with greater efficiency than WT ER α .

Previous studies have shown that ER α interacts with several members of the preinitiation complex that are required for transcription by RNA polymerase II, as well as with specific receptor coactivators that modulate and integrate transcriptional activity ²⁸. Since the K303R

ER α mutation does not appear to change in hormone- or DNA-binding affinities, we next hypothesized that this mutation might affect its ability to interact with components of the transcriptional machinery. We first assessed whether WT and mutant receptors differ in binding to the preinitiation complex, specifically to the TFIIB and TBP proteins that are involved in the early events of preinitiation complex assembly and are known to directly interact with ER α ^{29, 30}. The ability of the two receptors to physically interact *in vitro* with TFIIB and TBP was evaluated using GST pull-down assays. GST fusion proteins of TFIIB and TBP were incubated with ³⁵S-labeled, *in vitro* translated WT or mutant ER α 's in the absence or presence of estradiol. We saw that WT ER α interacts with TFIIB (Figure 3D) and TBP (Figure 3E) in both the absence and the presence of estradiol, as has been previously reported by others ^{29, 30}. The mutant ER showed no apparent differences in its interaction with either TFIIB or TBP protein, indicating that the K303R mutation does not alter binding to these proteins of the basal transcriptional machinery, as compared to its altered binding to AIB1 and SRC-2.

Corepressors inhibit WT-ER α transactivation but not mutant receptor

To analyze the capacity of corepressors to inhibit the transactivation, we tested the ability of NCoR and BRCA1 to affect WT and mutant ER α transactivation. HeLa cells transiently transfected with WT or mutant ER α demonstrated a significant increase in ERE-luciferase activity following estrogen treatment. In the presence of NCoR, cells expressing the WT ER α had a significant reduction in ER α activity (Figure 4A, compare bars 2 and 4, $p=0.0067$, t-test). In contrast, cells expressing the mutant receptor did not show a reduction in ER α transactivation in the presence of NCoR (Figure 4A, compare bars 6 and 8). Additionally, the ability of BRCA1 to reduce ER α transactivation demonstrated similar results with the mutant receptor. T47D cells transiently transfected with BRCA1 and WT ER α had a significant reduction in estrogen-induced transactivation (Figure 4B, compare bars 2 and 4, $p=0.0005$, t-test), whereas T47D cells expressing the mutant ER α did not (Figure 4B, compare bars 6 and 8, $p=0.5$, t-test). Collectively, these data demonstrate a reduced ability of corepressors to inhibit estrogen-mediated activity of the mutant ER α .

ER α promoter dynamics

Previous reports have demonstrated that ER α and its cofactors cycle on and off of the promoter in a dynamic manner ^{14–16}. GST-pull down experiments demonstrated increased binding of ER α and the SRC family of coactivators to the mutant, therefore we wanted to determine if cofactor dynamics of the pS2 promoter were altered in the presence of the K303R ER α mutation. CHIP analysis was performed for ER α , SRC-3, and p300 binding on the pS2 promoter following estrogen treatment. MCF-7 cells stably expressing the WT or K303R-ER α were subjected to CHIP analysis following serum starvation and estrogen treatment for the indicated timepoints. WT ER α -expressing cells demonstrated a cycle time of approximately 120 minutes, with delineated two cycles in a 300 minute assay (Figure 5A). In contrast, K303R ER α expressing cells had an initial increase in ER α on the pS2 promoter, but did not demonstrate an “off” cycle at 120 minutes (Figure 5A). Additionally, this initial “on” cycle of the K303R ER α was reduced when compared to WT-ER α expressing cells. Further analysis of the dynamics with the K303R mutant receptor revealed that despite the lack of an “off” cycle, the second “on” cycle occurred at a similar timepoint

as WT ER α , and this second cycle had a higher amplitude, with a longer residence time on the pS2 promoter when compared with WT ER α (Figure 5A). Collectively, these data demonstrate that WT ER α has promoter cycling dynamics similar to previously published results, and the mutant K303R ER α demonstrated a reduced initial cycle followed by a larger second cycle.

Analysis of SRC-3 occupancy on the pS2 promoter in WT ER α -expressing cells demonstrated an initial cycle with a peak at 60 minutes followed by a second peak at 120 minutes (Figure 5B). Additionally this second cycle had increased amplitude and a longer “on” time compared with the first peak (Figure 5B). Cells expressing the K303R ER α mutant demonstrated maximal interaction with the pS2 promoter at 120 minutes (Figure 5B). In contrast to the WT ER α -expressing cells, a suggestion of cycling was observed, but the values never reached basal levels and the amount of mutant ER α present on the promoter was always elevated compared to WT ER α expressing cells (Figure 5B). To confirm that the presence of the K303R ER α protein altered coactivator dynamics, the dynamics of p300 in the presence of WT ER α or mutant ER α were also analyzed. In the presence of WT receptor p300 protein occupancy on the pS2 promoter initially peaked at 30 minutes followed by a return to basal levels, and then a second peak at 150 minutes post estrogen stimulation (Figure 5C). K303R ER α expressing cells demonstrated a longer cycle time with an initial peak at 90 minutes, followed by a return to basal levels (Figure 5C). The second peak in the K303R ER α expressing cells had a higher amplitude compared to the first peak. When compared with WT ER α , this second peak occurred later and was also elevated (Figure 5C). Collectively, these data demonstrate that in the presence of the mutant K303R ER α cells display increased coactivator interactions on the pS2 promoter.

The K303R ER α mutation does not create a new coregulator binding site

We observed an increased interaction between the SRC family of coactivators and the mutant receptor. However the reported interaction site for TIF-2 on ER α is residues 539–544¹⁸. To determine if the K303R ER α mutation created a new binding site within the hinge domain, we performed GST-pull down experiments with a number of truncated ER α constructs. As expected, the ER α fragment containing residues 263–595 encompassing the hinge and hormone binding domain bound TIF-2 in the presence of estrogen (Figure 6, lane 2 vs lane 3). When only the hinge domain was used, no binding was observed for either the WT or mutant receptor (Figure 6, lanes 4–7), thus demonstrating that the increased binding of ER α to TIF-2 was not due to the creation of a new TIF-2 binding site.

The ER α F domain plays an important role in ER α regulation

It has previously been shown that regions within the F domain of ER α , in part, serve to modulate its transcriptional activity, including the effects of coactivators, agonist, and antagonist^{31–33}. Additionally, Richards et al have suggested that removing the F domain shifts the equilibrium of ER α toward a more active state in the absence of ligand³⁴. Recently Koide et al have shown that residues immediately distal to Q580 play an important role in the regulatory function of the F domain³⁵, and TIF-2 binds to ER α within the F domain. Therefore, we wanted to determine if the modulatory function of the F domain was altered in the presence of the K303R mutation. Transactivation assays were analyzed in the

presence or absence of exogenous TIF-2 to determine the ability of TIF-2 to coactivate transactivation of serial deletion mutants of the F domain. GAL4-DBD fusion constructs of WT ER α and K303R ER α fragments were examined using a GAL4 binding site, and a TATA promoter-luciferase reporter in the presence of estrogen. ER α constructs containing the amino acids 251–595 (hinge and regions E-F), 251–577 (hinge, region E and F truncation), 251–566 (hinge, region E and F truncation), and 251–554 (hinge and region E) were used.

As expected, both the WT and mutant ER α 251–595 constructs demonstrated estrogen-induced coactivation that was increased in the presence of TIF-2, thus indicating that TIF-2 can serve to effectively coactivate ER α activity in this model system. Additionally, the mutant-containing construct demonstrated increased activity in both the presence and absence of TIF-2 when compared with WT ER α . When the WT ER α construct was truncated at amino acid 577, the basal level of activation was increased to a level similar to the mutant receptor. Furthermore, TIF-2 coexpression only resulted in a small increase in coactivation of WT ER α . Analysis of the deletion constructs WT-ER α 251–566 and 251–554 demonstrated that in the absence of TIF-2, transactivation was increased above the full-length, but to similar levels as to the 577 truncation. While the 577 truncation only resulted in a small increase in TIF-2 induced coactivation, the 566 and 554 truncations allowed for a greater coactivation by TIF-2. In contrast, the deletion constructs 251–577 and 251–566 containing the K303R mutation all demonstrated similar results as the full-length K303R construct. However, the K303R 251–554 deletion construct demonstrated a small increase in transactivation compared with the additional three constructs analyzed. Collectively, these data demonstrate that amino acids 577–595 contain an important regulatory domain for estrogen-induced transactivation of WT-ER α . Additionally, while all constructs demonstrated TIF-2 induced coactivation, amino acids 566–595 contain a regulatory domain that plays a role in the levels of coactivation by TIF-2. Since the K303R mutant ER α construct was only marginally affected by deletion of these regulatory domains, it suggests that the presence of this K303R mutation within the hinge domain alters the ability of the F domain to regulate ER α transactivation. These data suggest that the hinge domain plays an important role in regulating the ability of the F domain to modulate ER α transactivation.

Discussion

As we have demonstrated previously, the mutant K303R ER α is a better substrate for phosphorylation by PKA at S305¹⁰, and S305 has been shown to be a PAK-1 phosphorylation site⁹. Additionally, K302/K303 is an important acetylation site and is regulated, in part, by the phosphorylation status of S305¹⁰. Furthermore, the K303 site can be further regulated by sumoylation, thereby influencing WT ER α transactivation³⁶. It is clear that this lysine is involved in sumoylation, acetylation, and regulation of S305 phosphorylation, thus it is a major site of ER α posttranslational modifications, of which, the consequences can be seen in the altered coregulator interactions. While cofactors such as SRC-1, 2, and 3, demonstrated increased binding, TBP and TFIIB did not demonstrate altered binding. Additionally, transactivation analyses have previously shown that MTA-1 can repress the activity of both WT and mutant ER α ¹², and MTA-2 can only suppress the activity of WT-ER α and not the mutant ER α ¹³. It is important to reiterate that not all

cofactors tested were affected by the K303R ER α mutation, thus demonstrating specific cofactor regulation rather than affecting the global signaling of the protein. Here we show that both BRCA-1 and NCoR have the ability to repress the activity of WT-ER α , but not the mutant ER α (Figure 6). Furthermore, corepressors demonstrated differences in activity between the WT and mutant ER α proteins. Collectively, these data demonstrate that the 303 lysine is an important site of posttranslational regulation and its mutation to arginine can significantly alter the proteins interactions with coregulatory proteins.

The F domain of ER α has an important role in the regulation of ER α ligand responses. While many studies have analyzed the F domain in response to various agonist and antagonist ligands, the results are not always consistent. Recently, Koide et al have published results demonstrating that deletion of the entire F domain from full-length ER α reduced the ability of ER α to increase transactivation in response to estrogen³³. Previous results from the same laboratory have shown that deletion of the F domain did not affect estrogen-,induced transactivation of ER α ³⁷ thus demonstrating complex regulation by the F domain. The previous two studies analyzed the full-length (minus the F domain) receptor in Hela cells. An additional study utilizing amino-terminal truncated forms of ER α in a yeast assay demonstrated that the F domain was inhibitory to estrogen-induced ER α transactivation³⁸. While the recent studies by Koide et al demonstrated reduced transactivation in F domain truncation mutants, they further narrowed the important region to amino acid residues 579–584³³. This is interesting because our studies reported here, also demonstrate that amino acids 577–595, and 577-566, play important roles in the regulation of WT-ER α transactivation. First, deletion of the amino acids 577–595 increased estrogen-induced transactivation, but did not have a great impact on ability of the coactivator TIF-2 of affect transactivation. In contrast, the further deletion of residues 566–595 significantly increased the ability of TIF-2 to coactivate WT ER α transactivation. While previous studies using full-length ER α have demonstrated no effect³⁷ or reduced³³ ER α transactivation when the F domain was deleted, our studies herein demonstrate that the F domain acts to inhibit estrogen-induced transactivation and TIF-2 coactivation of ER α . Our studies utilized an amino truncated form of ER α beginning at amino acid 251, thus removing the ligand-independent AF-1 domain to allow us to study the F domain functions in the absence of inherent ligand-independent transactivation. Our studies demonstrating an AF-2 inhibitory region within the F domain are in agreement with previously published results showing that in the absence of the AF-1 region, the F domain reduces estrogen-induced transactivation³⁸. Thus, the F domain may act in concert with the AF-1 region, to regulate the ligand-dependent AF-2 transactivation.

While the F domain contains a regulatory region, it appears that the K303R ER α mutation results in a loss of this regulation. In Figure 7, serial truncations of the F domain only resulted in increased ER α transactivation when the full F domain was deleted, the increase was modest. In contrast, all truncated constructs analyzed led to an altered transactivation by WT ER α . Collectively, these data demonstrate that the F domain has important roles for both ligand and coactivator-induced transactivation for WT ER α . Additionally, as the K303R ER α only demonstrated a marginal response to F domain deletion, and deletion of the F domain in WT ER α caused the WT ER α to behave similarly to K303R ER α , these

data suggest that the regulatory units within the F domain may play a role in the hypersensitive phenotype of K303R ER α expressing cells.

Studies evaluating ER α and coactivators on promoters of target genes have shown that these proteins cycle “on” and “off” promoters, and estrogen treatment leads to elongated promoter cycling and increased amplitudes of each cycle¹⁴. Additionally, when cells were treated with the proteasome inhibitor MG-132 to reduce regulated turnover, promoter occupancy was reduced and the “off” phase of the cycle was significantly elongated, thus demonstrating that increased promoter activity is associated with increased amplitude and elongated cycles, whereas reduced activity is associated with an elongated “off” cycle and reduced amplitudes. Based on this data, we would expect the hypersensitive K303R ER α to demonstrate an elongated cycle time and increased amplitude. Here we demonstrate that WT ER α expressing cells demonstrated similar cycling dynamics as those previously reported. However the K303R ER α expressing cells demonstrated altered dynamics with the amplitude of the initial cycle is reduced whereas the amplitude of the second cycle is increased. Furthermore, two coactivators, AIB1 and p300 also demonstrated altered dynamics with increased amplitude and elongated cycle times. Thus, these coactivators confirm the changes observed in promoter dynamics of the K303R ER α . The increased amplitudes and elongated “on” phase of the cycles alone would suggest increased signaling by the K303R mutant receptor, combined with the increased interactions with coactivator proteins and the hypersensitive phenotype previously reported.

Tumors grown as xenografts in nude mice displayed an increased ability of K303R ER α cells to grow in the presence of low estrogen concentrations. Additionally, when estrogen was withdrawn, 50% of K303R ER α experimental tumors displayed estrogen independence in approximately one year time. In contrast, no WT ER α -expressing cells gained estrogen independence during this period. *In vitro*, when MCF-7 cells are grown in the absence of any estrogen stimulation, estrogen independence eventually develops and the cells will also form tumors in the presence of low estrogen concentrations⁴. In contrast to this data, only the mutant ER α -expressing cells demonstrated an ability to develop estrogen independence *in vivo*. Cyclin D1 and phospho-MAPK were analyzed as markers of proliferation in the wild-type vs. K303R ER α cells. Our initial analyses did not reveal any differences in these two proteins. Thus, thorough study of the potentially activated mechanisms of tumor growth is warranted and will require a more detailed analysis that is beyond the scope of this paper.

Here we have demonstrated an important role for the K303R ER α mutation in human breast cancer. The data would suggest that this particular mutation would demonstrate a more important role in the development and/or recurrence of breast cancer in an estrogen-independent manner. Indeed our recently published *in vitro* results suggest that the mutation could play a role in resistance to aromatase inhibitors³⁹. These data also demonstrate that the K303R ER α has increased transcriptional activation and a loss of the “normal” F domain-mediated regulatory mechanisms inherent in the wild-type ER α , it would not be surprising if this mutant receptor also plays an important role in the development and progression of estrogen responsive breast cancer.

Acknowledgments

MHH was supported by DAMD17-03-1-0417, and this work was supported by NIH/NCI and CA72038 to SAWF

Abbreviations

ERα	estrogen receptor α
IB	immunoblot
IP	immunoprecipitate
pY	phospho-tyrosine
sFBS	charcoal-stripped FBS

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A)

B)

C)

Figure 1. Growth of wild-type ER α and K303R ER α cells *in vivo*

A) Cells were injected into nude mice in the presence of 80 pg/ml or 300 pg/ml of circulating estrogen. Tumors were measured twice weekly and time to reach 300 mm³ is plotted ($p=0.0011$, chi-square). B) Three tumors from each group were analyzed by Western blot as described in Materials and Methods. Shown above the panel is the approximated concentration of circulating estrogen. C) Tumors were initiated in the presence of 300 pg/ml of circulating estrogen and when the tumors reached 200 mm³ the estrogen was removed and the time to tumor regression is plotted ($p=0.0185$, chi-square).

Figure 2. The K303R mutation does not affect hormone binding affinities

Full-length WT or mutant receptors were synthesized *in vitro* and incubated for 2 hours in the presence of [2,4,6,7-³H]-17β-estradiol with or without a 200-fold excess of unlabelled estradiol. Unbound steroids were removed using dextran-coated charcoal. Bound steroid was measured by scintillation counting and specific binding was calculated after subtraction of non-specific binding. Representative Scatchard plots for wild-type (A) and K303R ERα (B) are shown.

D)

E)

Figure 3. Protein interactions of WT and K303R ERα

A) Full-length coactivators were synthesized *in vitro* in the presence of [³⁵S]-methionine and then incubated with Sepharose beads containing immobilized glutathione, GST-WT ERα, and GST-K303R ERα in the absence or presence of estradiol. Bound coactivators were eluted and separated by SDS-PAGE followed by autoradiography. Increasing levels of estradiol were used (4×10^{-8} , and 5×10^{-8} , 6×10^{-8} , 7×10^{-8} , and 1×10^{-6} M). B). HeLa cells were transiently transfected with wild-type or mutant ERα and treated with 10^{-11} or 10^{-9} estradiol for 24 hours prior to lysis. SRC-3 complexes were immunoprecipitated with a specific antibody and immunoblotted for SRC-3 and ERα, quantitation of the blot is shown in the panel. D and E) GST-pulldown assays were performed as previously described¹³ with the exception of GST-TBP and GST-TFIIB were immobilized on Sepharose beads to capture ERα. Bound receptors were eluted and separated by SDS-PAGE followed by autoradiography. Increasing levels of estradiol were used 1×10^{-10} , and 1×10^{-6} M for TBP (D), as well as 1×10^{-11} , 1×10^{-10} , 1×10^{-8} , and 1×10^{-6} M for TFIIB (E).

A)

B)

Transactivation Assays

Figure 4. NCoR and BRCA1 are unable to corepress K303R ER α transactivation

Cells were transiently transfected and serum starved for 24 hours prior to estrogen treatment [10^{-9} M] for an additional 18–24 hours. Luciferase activity was measured and normalized to co-transfected β -galactosidase as described in Materials and Methods. A) HeLa cells were transiently transfected with and ERE-Luciferase and WT ER α or K303R ER α in the presence or absence of NCoR. B) T47D cells were transiently transfected with an ERE-Luciferase and WT or K303R ER α in the presence or absence of BRCA1, as indicated.

Figure 5. Mutant ER α displays altered dynamics on the promoter

Chromatin immunoprecipitation was performed on the pS2 for ER α (A), SRC-3 (B), and p300 (C) in WT ER α or K303R ER α cells, as indicated. Cells were treated with estrogen for the indicated timepoints, lysed, protein was immunoprecipitated, and the relevant sequence from the pS2 promoter was amplified by semi-quantitative PCR as described in Materials and Methods. Relative levels were quantitated and graphed.

Figure 6. The K303R ER α mutation does not create a new SRC-2 binding site
 GST pulldown was performed as previously described. Truncated ER α constructs (as indicated) were immobilized on Sepharose beads and bound SRC-2 was eluted, separated by SDS-PAGE and analyzed by autoradiography.

Figure 7. The F domain is involved in the regulation of ERα
GAL4 DBD fusion constructs of wild-type and K303R-ERα fragments 251–595, 251–577, 251–566 and 253–554 (F domain deleted) were prepared as described in Materials and Methods. Transactivation was analyzed using a GAL4 binding site and a TATA promoter-luciferase reporter. Estrogen [10⁻⁹ M] was added, in the absence of the presence of the TIF-2 coactivator. Data represents the mean of two individual experiments performed in triplicate relative to no added estrogen control.