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Griekspoor, A.C.

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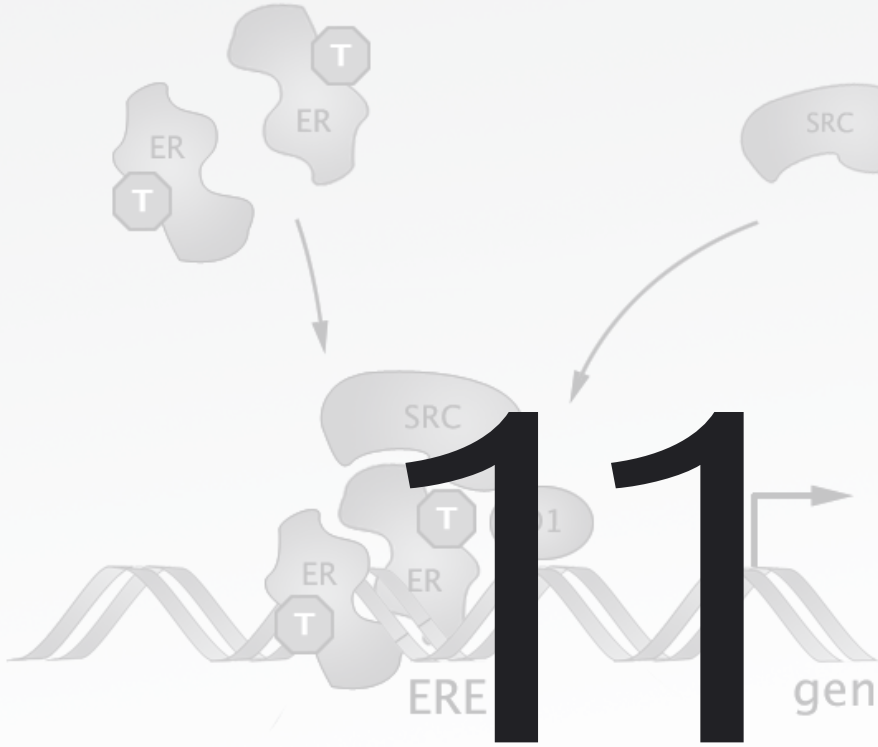

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Protein Kinase A-induced tamoxifen resistance through altered orientation of Estrogen Receptor α towards co-activator SRC-1

Wilbert Zwart[1], Alexander Griekspoor[1], Valeria Berno[2], Kim Lakeman[1], Jacco van Rheenen[3¶], Kees Jalink[3], Michael Mancini[2], Jacques Neefjes[1], and Rob Michalides[1]

[1] Division of Tumour Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.
[2] Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, Texas, USA.
[3] Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Resistance to tamoxifen treatment is observed in half of the recurrences in breast cancer where the anti-estrogen tamoxifen acquires agonistic properties for transactivating ERα. Using Fluorescence Resonance Energy Transfer, FRET, we demonstrate in this study that tamoxifen resistance due to phosphorylation of Serine 305 in ERα by Protein Kinase A results from an altered orientation between ERα and its coactivator SRC-1, which renders the transcription complex active in the presence of tamoxifen. This altered orientation between the C-termini of ERα and SRC-1 requires prolonged association between the AF-1 domain at the N-terminus of ERα and SRC-1. This intermolecular reorientation as induced by PKA or PAK1-mediated phosphorylation of ERα-Serine 305 combined with tamoxifen, provides a unique model for resistance to tamoxifen.

CORRESPONDENCE

Rob J. A. M. Michalides
Division of Tumour Biology
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands
Tel.: +31 20 512 2022
Fax: +31 20 512 2029
E-mail: r.michalides@nki.nl

Significance

Patients receive tamoxifen as treatment against estrogen receptor positive breast cancer, where in half of the recurrences the tumor acquires resistance against tamoxifen. Protein Kinase A- or PAK1-mediated phosphorylation of Serine 305 of ERα is responsible for resistance to tamoxifen. Here, we report on the mechanism of this form of tamoxifen resistance and demonstrate that phosphorylation of Serine 305 by Protein Kinase A in ERα leads to an altered orientation between ERα and its coactivator SRC-1, which renders the transcription complex active in the presence of tamoxifen.

¶ Present address: Albert Einstein College of Medicine, Department of Anatomy & Structural Biology, New York, NY, USA

Introduction

Tamoxifen is an effective anti-cancer drug in Estrogen Receptor alpha (ERα) positive breast cancer patients. In recurrent disease however, half of the patients still develop resistance where tamoxifen acquires agonistic properties for transactivation of ERα. Various mechanisms may account for resistance to tamoxifen, including activation of the Mitogen Activated Protein Kinase (MAPK), Protein Kinase A (PKA) and p21-activated kinase-1 (PAK-1) signaling pathways that show enhanced activity in tamoxifen-resistant breast tumors (1-3) and various phosphorylation events on ERα. However, the molecular details of how these events contribute to tamoxifen resistance remain elusive. Antagonists of ERα act by altering the orientation of C-terminally located α-helix 12 of the Ligand

Binding Domain (LBD) of ER (4). In the agonist-bound state, cofactors bind to the pocket composed of helices 3, 4, 5 and 12 (5). Anti-estrogens induce a distortion in α-helix 12 covering this binding pocket, thereby preventing the association with the p160 family of coactivators (6). These cofactors are mandatory for transcription to occur (7) and include SRC-1 (or NcoA-1), SRC-2 (also known as TIF-2, GRIP1 or NcoA-2) and SRC-3 (also known as RAC3, ACTR, AIB1, P/CIP or TRAM) (8). SRC-1 interacts at the one hand with CREB-binding protein (CBP), and at the other hand with both the N-terminal AF-1 and the C-terminal AF-2 domains of ERα (9). Both AF-1 and AF-2 domains cooperate in transactivation of ERα (10, 11). Activity of SRC-1 is modified by phosphorylation at multiple sites, two of which are attributed to MAPK activation (12). The SRCs have different properties: SRC-3 is only recruited to ERα upon binding to agonists (13), whereas SRC-1 is already associated with ERα under hormone-depleted conditions (14, 15). Overexpression of SRC-1 and SRC-3 is correlated with tamoxifen resistance in breast cancer patients (16, 17) and, moreover, agonistic activity of tamoxifen is enhanced by overexpression of SRC-1 in normal uterus tissue (18).

Previously we reported that tamoxifen resistance mediated by Protein Kinase A (PKA) is caused by phosphorylation of Serine 305 (S305) of ERα (3). Recently, S305 of ERα was also reported to be the target of p21-activated kinase-1, PAK-1 (19), and its overexpression correlates with resistance to tamoxifen in breast cancer patients (2). These findings identify S305 as a crucial site in ERα that upon phosphorylation by either PKA or PAK-1 is responsible for resistance to tamoxifen. This phosphorylation switches tamoxifen from an antagonist to an agonist of ERα. The mechanistical details of this process are, however, still unclear. Here, we report that PKA mediated phosphorylation of ERα alters the orientation of ERα towards coactivator SRC-1, without affecting overall binding between ERα and SRC-1 in tamoxifen treated cells. We applied Fluorescent Resonance Energy Transfer (FRET) to measure an altered orientation between ERα and SRC-1 that was dependent on phosphorylation of the PKA target S305 and was capable of recruiting RNA polymerase and induce transcription in cells treated with tamoxifen. In summary, tamoxifen resistance via PKA or PAK-1 occurs through phosphorylation of S305 in ERα, which alters its orientation towards coactivator SRC-1 rendering the complex transcriptionally active.

Experimental Procedures

Plasmids

Fluorescent constructs. For the construction of SRC-$1_{623\text{-}711}$-YFP a fragment of SRC-1 was amplified by PCR from pCMV-SRC-1 (20) (kind gift from Dr. Rene Bernards) using forward primer 5' GGAATTCACCACCATGCCCAAGA AGAAGAGAAAGGTGGACAGTAAATACTCTCAA and reverse primer 5' CCCAGCGGCCGCTTATCAGGCTC GACAGACAA, introducing restriction sites for *Eco*RI and *Not*I. This fragment was subsequently inserted in a pcDNA3-YFP vector. For construct SRC-1_{FL}-YFP, full-length SRC-1 was amplified from pCMV-SRC-1 by PCR with forward primer 5' CCCAAGATCTACCACCATGAGTGGCCTT GGGGACAGTT and reverse primer 5' CCCAGCGGCC GCATCAGTAGCTGCTGACGGAGG, introducing restriction sites for *Bgl*II and *Not*I and removing the stop codon. This fragment was subsequently inserted in a pcDNA3-YFP vector at these sites. $ER\alpha_{wt}$-CFP and $ER\alpha_{S305A}$-CFP were constructed as described previously (3).

Mammalian 2-Hybrid constructs. For the Gal4 transactivating and DNA binding domain (TA and DBD, respectively), the TA and DBD containing yeast 2-hybrid vectors pPC86 and pPC97 (Clonetech) were digested with *Hind*III and *Eco*RI, and were both inserted in a pcDNA3 vector. For the construct DBD-SRC-$1_{623\text{-}711}$-YFP, SRC-$1_{623\text{-}711}$-YFP was isolated by PCR from pCMV-SRC-1 (20) with forward primer 5' GGAATTCACCACCATGCCCAAGA AGAAGAGAAAGGTGGACAGTAAATACTCTCAA and reverse primer 5' CCCAGAATTCTCACTTGTACAGC TCGTCCATG, introducing restriction sites for *Eco*RI and *Xba*I, and was subsequently inserted in the DBD containing pcDNA3 vector. For the construct DBD-SRC-1_{FL}, SRC-1_{FL}-YFP was amplified by PCR from pCMV-SRC-1 with forward primer 5' CCCACCCGGGATGAGTGGCCTTGGG GACA and reverse primer 5' CCCATCTAGATTATTCAG TCAGTAGCTGCTGACGGA, producing restriction sites for *Sma*I and *Xba*I. This fragment was subsequently inserted in the *Xba*I and *Eco*RI sites of pcDNA3 vector, where the *Eco*RI site was filled up. For the TA-$ER\alpha_{wt}$ and TA-$ER_{\alpha S305A}$ constructs, fragments were generated from $ER\alpha_{wt}$-CFP or $ER\alpha_{S305A}$-CFP by PCR with forward primer 5' CGGAATT CAAATGACCCTCCACACCAAAGCATCT and reverse primer 5' CCCAACTAGTTCAGACTGTGGCAGGGAA ACCCT, introducing restriction sites for *Eco*RI and *Spe*I and inserted into the corresponding restriction sites of TA-containing pcDNA3 vector.

Mammalian 2-hybrid assay. U2OS cells were cultured in 12 well plates 96 hours prior to analysis. 24 hours later cells

were transfected using PEI (Polyethylenimine, MW 25kDa (Polysciences Inc., Germany)(21)) with TA-ERα_{wt} or TA-ERα_{S305A} (0.5µg), DBD-SRC-1-YFP (full length or aa 623-711) (0.5µg), GAL4 responsive Luciferase reporter construct (0.5µg) and ER-insensitive Renilla luciferase construct (1ng) as control for transfection efficiency (3). Four hours after transfection, medium was replaced with Dulbecco's medium without phenol red, supplemented with 5% Charcoal Treated Serum (CTS, Hyclone) and cultured in the absence or presence of E2 or 4'OH-tamoxifen (Sigma-Aldrich, Steinheim, Germany) at a final concentration of 1 µM. 24 hours prior to analysis, medium with (anti-)estrogens was replaced and 8Br-cAMP (Sigma-Aldrich) was added where indicated at a final concentration of 100 µM. Luciferase activity was determined as described previously (22).

Fluorescence Recovery After Photobleaching (FRAP) and Fluorecence Loss in Photobleaching (FLIP)

U2OS cells were cultured on coverslips for 48 h before treatment in CTS containing medium. 24 hours prior to imaging, cells were transfected with a combination of ERα_{wt}-CFP, SRC-1_{FL}-YFP or SRC-$1_{623-711}$-YFP. After transfection medium was replaced with Dulbecco's medium without phenol red, supplemented with CTS. Prior to analysis, coverslips were placed in 2 ml bicarbonate-buffered saline (140 mM NaCl, 5mM KCl_2, 2mM $MgCl_2$, 1mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM D-Glucose, 10 mM Hepes pH 7.3) and analyzed in a heated tissue culture chamber at 37°C under 5% CO_2. Where indicated cells were pretreated with 10 µM forskolin for 15 min, and subsequently treated with E2, 4'OH-tamoxifen or left untreated. Images were acquired on a TCS-SP2 confocal microscope (Leica, Mannheim, Germany), using a 63x oil emersion objective. Fluorescence intensities were measured at the bleach spot (FRAP) and at the far end of the nucleus (FLIP). Zoom factor was set at 4x for all experiments. $t_{½}$ recovery value was calculated by $t_{½} = F_{FRAP}\infty - F_{BLEACH} /2$, where ∞ is the asymptotic value of the corresponding curve, as described before (23). Immobile fraction (IF) was calculated as $IF = ((F_{FLIP}\infty - F_{BLEACH}) - (F_{FRAP}\infty - F_{BLEACH}))/ (F_{FLIP}\infty - F_{BLEACH}) \times 100\%$, where $F_{FLIP}\infty$ and $F_{FRAP}\infty$ are the fluorescence plateau levels of the corresponding curves and F_{BLEACH} the fluorescence level immediately after bleach.

Confocal (CLSM) FRET imaging by sensitized emission

U2OS or HeLa cells were cultured on coverslips for 48 hours before imaging. 24 hours prior to imaging cells were transfected with the constructs indicated and medium was replaced with Dulbecco's medium without phenol red, supplemented with CTS. Mel JuSo cells, stably transfected with pcDNA3 constructs containing only CFP or YFP were included to the culture for leak-through corrections and internal controls. Coverslips were placed in 2 ml bicarbonate-buffered saline and analyzed in a heated tissue culture chamber at 37°C under 5% CO_2. Where indicated forskolin was added at a final concentration of 10 µM for 15'. FRET between CFP and YFP molecules was determined by calculating the sensitized emission (the YFP emission upon CFP excitation) using separately acquired donor and acceptor images as described previously (24). In short, images were acquired on a TCS-SP2 confocal microscope (Leica, Mannheim, Germany). Three images were collected: CFP excited at 430nm and detected between 470 and 490nm; indirect YFP: excited at 430nm and detected between 528 and 603nm; and direct YFP excited at 514nm, and detected between 528 and 603nm. Because of considerable overlap of CFP and YFP spectra, YFP emission was corrected for leak-through of CFP emission, and for direct excitation of YFP during CFP excitation. FRET was calculated using correction factors obtained from cells expressing either CFP or YFP alone, which were included for every image, as described before (25). Then the apparent donor FRET efficiency (E_D) was calculated by relating the FRET to the total emission of the donor cell, after which the E_D picture was overlayed with a false color look up table. Using these methods, differences in FRET efficiency can be measured with an accuracy of 0.5% (24). For graphic representation, the E_D was calculated for each pixel from the raw data files of the represented cell, and was exported to Microsoft Excel. Here, the amount of pixels was related to the corresponding E_D, and plotted in a histogram (24).

RNA Polymerase II recruitment assay

For CLSM analysis prolactin promoter/enhancer (PRL) array containing HeLa cells (26) were cultured in Dulbecco's medium containing CTS and were transfected with ERα_{wt}-CFP or ERα_{S305A}-CFP and SRC-1_{FL}-YFP using PEI. After 4 hours the medium was replaced with Dulbecco's medium without phenol red supplemented with 5% CTS. Cells were cultured in CTS only or supplemented with 10 µM forskolin for 15 min. Subsequently, cells were treated for 2 hours with 1µM Estradiol, 1 µM 4'OH-tamoxifen or left untreated, thereafter fixed with 3.7% formaldehyde in PBS and subsequently stained with anti-RNA polymerase II antibody 8WG16 (Covanche Research Products, Inc.) and secondary antibodies conjugated to Alexa 633 (Molecular Probes, Leiden, The Netherlands). Images were taken with a Leica TCS SP2 System equipped with a 63 x oil emersion objective. CFP was excited at 458 nm, and emission measured at 460-500 nm. YFP was excited at 514 nm, emission measured at 528-600 nm. Alexa 633 was excited at 633 nm, emission measured at 645-720 nm.

Quantitative RT-PCR and RNA FISH

Prolactin promoter/enhancer (PRL-) array containing HeLa cells (26) were transfected with $ERα_{wt}$-CFP or $ERα_{S305A}$-CFP using electroporation, and subsequently cultured in Dulbecco's medium containing CTS. Immediately after seeding the cells, 1 μM tamoxifen was added or the cells were left untreated. After 6 hours, 8-Br-cAMP was added where indicated at a final concentration of 100 μM. After 16 hours, cells were lysed and RNA was extracted using Trizol (Invitrogen) according to the manufacturers protocol. RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen), on which QPCR was performed using CYBR Green (Applied Biosystems), according to the manufacturers protocols. The DsRed cDNA was amplified with forward primer 5' CCAGTTCCAGTACGGCTCCA and reverse primer 5' GCCGTCCTCGAAGTTCATCA. As a control, the observed DsRed signal was related to beta actin RNA levels, using a forward primer 5' CCTGGCACCCAGCACAAT and reverse primer 5' GGGCCGGACTCGTCATACT.

RNA FISH was performed as described previously (26). PRL DNA array containing HeLa cells were transfected with either CFP-$ERα_{wt}$ or CFP-$ERα_{S305A}$ and treated 2 hours before analysis with forskolin for 15 minutes, followed by 1μM 4'OH-tamoxifen or treated with 4'OH-tamoxifen only. The cells were stained with an antibody against ERα and RNA FISH for DsRed transcripts was performed as described previously (26). Informative arrays of large enough size were analyzed for the Alexa-546 signal intensity of DsRed transcripts.

Results

Phosphorylation of ERα-Serine 305 by PKA does not influence mobility of SRC-1 and overall binding to SRC-1.

Since activation of ERα requires binding to a co-activator, we hypothesized that resistance to tamoxifen by PKA-mediated phosphorylation of Serine 305 (S305) would affect the binding between ERα and cofactor SRC-1. We investigated this first in a mammalian 2-hybrid (M2H) assay and used a SRC-1 truncation mutant, aa 623-711 ($SRC\text{-}1_{623\text{-}711}$) that only binds to the AF-2 domain of ERα as described previously (14), as well as the full length SRC-1 ($SRC\text{-}1_{FL}$), which interacts with both AF-1 and AF-2 domains of ERα (9), to distinguish between the interaction of SRC-1 with AF-1 and/or AF-2 domains of ERα. Expression of the fusion proteins was confirmed by Western blotting (data not shown). Chimeras of

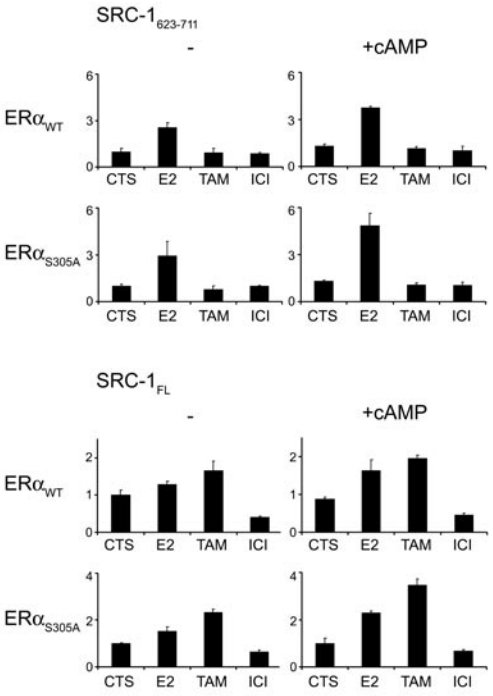

Figure 1. Binding between ERα and SRC-1 is not affected by PKA activation. U2OS cells transfected with DBD-SRC-1 (623-711 or full length), TA-ERα (wt or S305A), GAL4-luciferase and Renilla luciferase were cultured in CTS containing medium only, or in the presence of 1 μM E2 or 1 μM 4'OH-Tamoxifen for 96 hours. 24 hours prior to analysis 100 μM 8-Br-cAMP was added where indicated. Luciferase activity was measured and related to CTS values, which was set to 1. Bars indicate standard deviations from 3 independent experiments.

ERα and the transactivation domain of GAL4 (TA-ERα), as well as $SRC\text{-}1_{623\text{-}711}$ or $SRC\text{-}1_{FL}$ fused to the DNA binding domain of GAL4 (DBD-SRC-1) were co-transfected with a GAL4-responsive luciferase expression construct and Renilla luciferase construct as a control (**Figure 1**). In this assay luciferase activity is directly related to the binding between the two fusion proteins. $SRC\text{-}1_{623\text{-}711}$ interacted with ERα in the presence of E2 and this was only slightly increased by preincubation of the cells with 8-Br-cAMP, which stimulated PKA activity (**Figure 1**, top panel). No binding was observed under tamoxifen and ICI 182,780 conditions, also not in the presence of 8-Br-cAMP. The binding of full length SRC-1 was increased by E2 and, surprisingly, also by antagonist tamoxifen (**Figure 1**, bottom panel). ICI 182,780 treatment resulted in a loss in binding. The interac-

tion between ERα and SRC-1_{FL} was not influenced by 8-Br-cAMP under all tested conditions. These results indicated that PKA activation does not affect the overall binding between ERα and SRC-1. In addition, whereas binding of SRC-1 to the AF-2 domain of ERα is rigid only in the presence of E2, the binding of SRC-1 to the AF-1 domain of ERα occurs also under hormone depleted and tamoxifen conditions, as was reported previously (26).

In order to verify the M2H results in a dynamic context and in living cells, we studied interactions between ERα and SRC-1 using Fluorescence Recovery After Photobleaching (FRAP) in combination with Fluorescent Loss In Photobleaching (FLIP), as illustrated in **Figure 2A**. In FRAP, all fluorophores in the region of interest are destroyed using a high intensity laser beam and recurrence of fluorescence is followed in time. The mobility of the fluorophore-tagged protein of interest, either freely diffusing or in complex with interaction partners, can be monitored in this way. In FLIP, the loss of fluorescence at a distant region in the nucleus from the bleach spot provides information about protein mobility from this region towards the FRAP spot. The difference in the steady state fluorescence intensities of the FLIP and FRAP curves is defined as the immobile fraction of the tagged protein. A representative FRAP-FLIP result with YFP-tagged SRC-$1_{623-711}$ in E2 and tamoxifen treated cells is shown in **Figure 2A**, with a summary of the $t_{½}$ recovery values in **Figure 2B**. The mobility of SRC-$1_{623-711}$ when bound to the AF-2 domain of ERα was in the presence of E2 less than under hormone-depleted conditions, indicating complex formation when ERα is E2 bound. The $t_{½}$ recovery value of the complex under conditions of CTS was higher than in the presence of tamoxifen, and was not affected by treatment with the PKA activator forskolin. Part of the SRC-$1_{623-711}$ molecules was immobile in the nucleus under conditions of CTS and E2. This in contrast to tamoxifen treated cells where all of the SRC-$1_{623-711}$ molecules diffused freely and no complex formation with ERα was observed. 40% of the YFP-tagged full-length SRC-1_{FL} was immobile, indicating that the exogenous SRC-1_{FL} is present in relatively low amounts. When in excess, most of the SRC-1_{FL} would be freely diffusing, whereas in limiting amounts, most of it would presumably be immobile. The $t_{½}$ recovery values for SRC-1_{FL} in the remaining mobile fraction indicated complex formation of SRC-1_{FL} under conditions of CTS, E2, and tamoxifen as well, since they were higher than the $t_{½}$ recovery value of YFP- SRC-1_{FL} alone (**Figure 2B**).

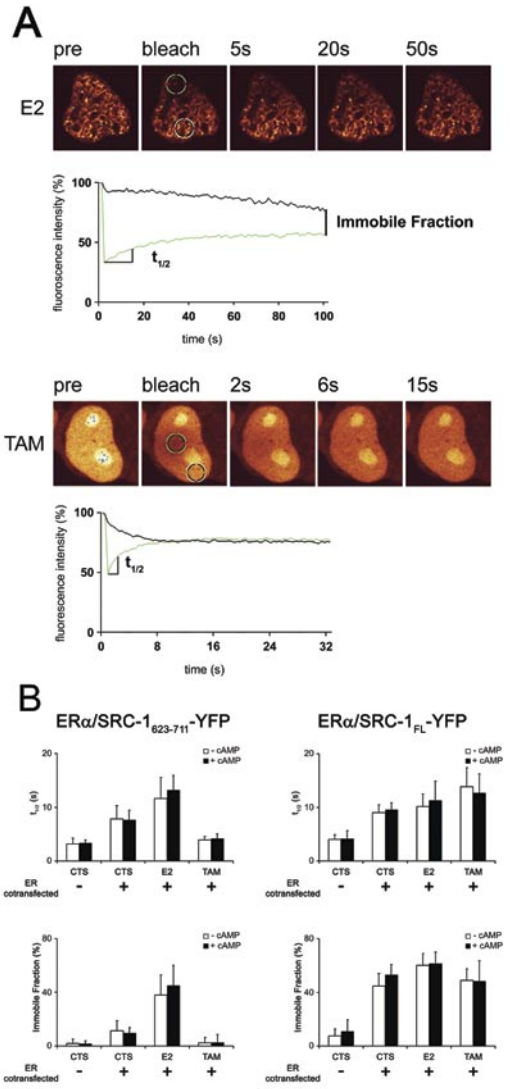

Figure 2. SRC-1 complex formation is unaltered by activation of PKA. **(A)** Example of FRAP and FLIP measurements. U2OS cells transfected with SRC-$1_{623-711}$-YFP and ERα-CFP were cultured in CTS containing medium that was supplemented with 1μM E2 (top) or 1 μM 4'OH-Tamoxifen (bottom). YFP was bleached and fluorescence intensities were followed in time in the bleach spot (green) and at the far end of the same nucleus (black), from which $t_{½}$ (half-time to recovery) and Immobile Fraction (= difference between FRAP and FLIP) were calculated as described in Materials and Methods.
(B) Transfectants as described in (A) or with SRC-1_{FL}-YFP and ERα-CFP were treated with E2 or 4'OH-Tamoxifen for 15 min or left untreated. Cells were pretreated with 10 μM forskolin for 15 min where indicated. Bars indicate standard deviation from 3 independent experiments, n >40 cells.

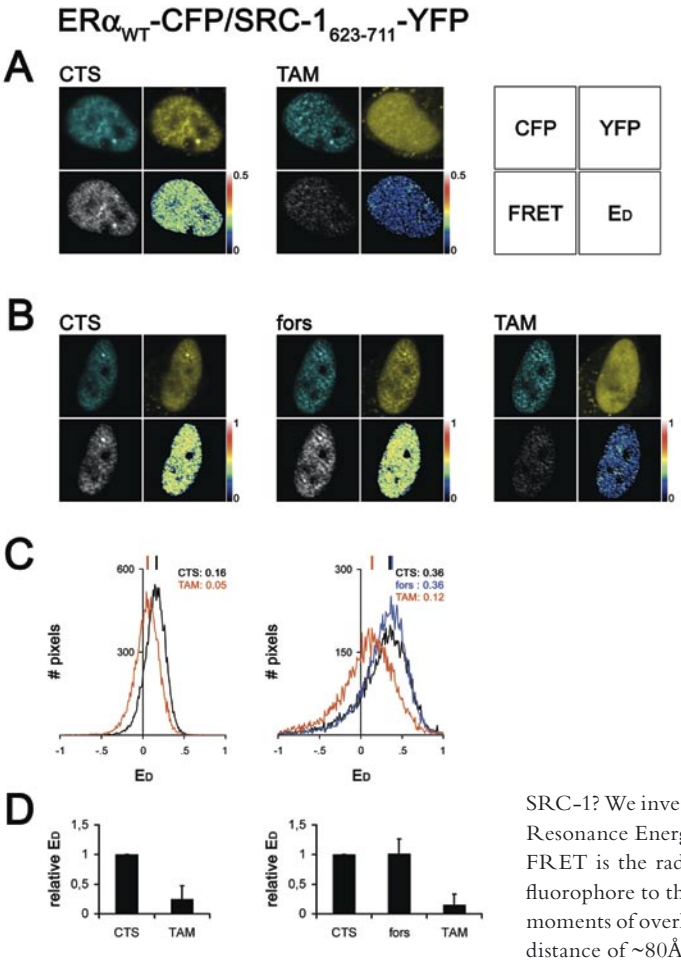

Figure 3. Orientation between ERα and SRC-$1_{623-711}$ is altered in the presence of tamoxifen and is independent of phophorylation of Serine 305 of ERα by PKA. FRET measurements were performed on PRL DNA array containing HeLa cells, transfected with ERα_{WT}-CFP and SRC-$1_{623-711}$-YFP **(A, B)**. FRET images were generated from cells cultured in CTS medium only (A and B, left panels) or after treatment with 10 mM forskolin prior to FRET measurements (B, middle panel). Subsequently, 1 μM 4'OH-tamoxifen (TAM) was added and after 15 min cells were imaged (A and B, right panels). FRET and donor FRET efficiency (E_D) were calculated as described in Materials and Methods. For each condition described in A and B, the raw E_D values from the whole nucleus were related to the total amount of pixels **(C)**. Mean E_D is indicated and quantifications **(D)** are done on >10 independent measurements, where the E_D under CTS conditions is set to 1 for each experiment (relative E_D). Bars indicate standard deviations.

The combined results of the M2H and FRAP experiments indicate that SRC-1_{FL} binds to ERα in CTS, as well as in E2 and tamoxifen treated cells. They strongly suggest that the AF-1 domain of ERα is involved in binding under all three conditions, whereas the AF-2 domain is participating in binding only in the presence of E2. Importantly, PKA activation, which is known to induce tamoxifen resistance, did not influence the interaction between SRC-1 and ERα in these assays, which measure overall binding without assessing the nature of the interaction.

Phosphorylation of S305 by PKA alters the orientation between ERα and SRC-1 under tamoxifen conditions.

If binding is not affected by PKA-mediated phosphorylation of S305, does this phosphorylation have an effect on the mode of interaction between ERα and SRC-1? We investigated this by FRET (Fluorescence Resonance Energy Transfer) using confocal imaging. FRET is the radiationless energy transfer from one fluorophore to the next, and occurs when two dipole moments of overlapping fluorophores couple within a distance of ~80Å. FRET is strictly dependent on distance between the fluorophores as well as their relative orientation, allowing visualization of conformational changes within protein complexes. FRET was detected between ERα-CFP and SRC-$1_{623-711}$-YFP (**Figure 3A**); excitation of CFP at 430nm yielded emission of YFP that, after correction of leak-through, only could have arisen from FRET (lower left panel). The observed FRET signal was corrected for the amount of CFP emission as is described in Materials and Methods, yielding the corrected FRET efficiency (E_D) that is presented in the lower right panel. E_D represents FRET independent of donor fluorescence. Under hormone-depleted conditions a high FRET efficiency was observed for ERα-CFP and SRC-$1_{623-711}$-YFP, which was strongly reduced by tamoxifen (**Figure 3A**), indicating that the interaction in CTS between ERα and SRC-$1_{623-711}$ was abrogated in the presence of tamoxifen. When FRET efficiency was quantified by determining the E_D per pixel as described in Materials

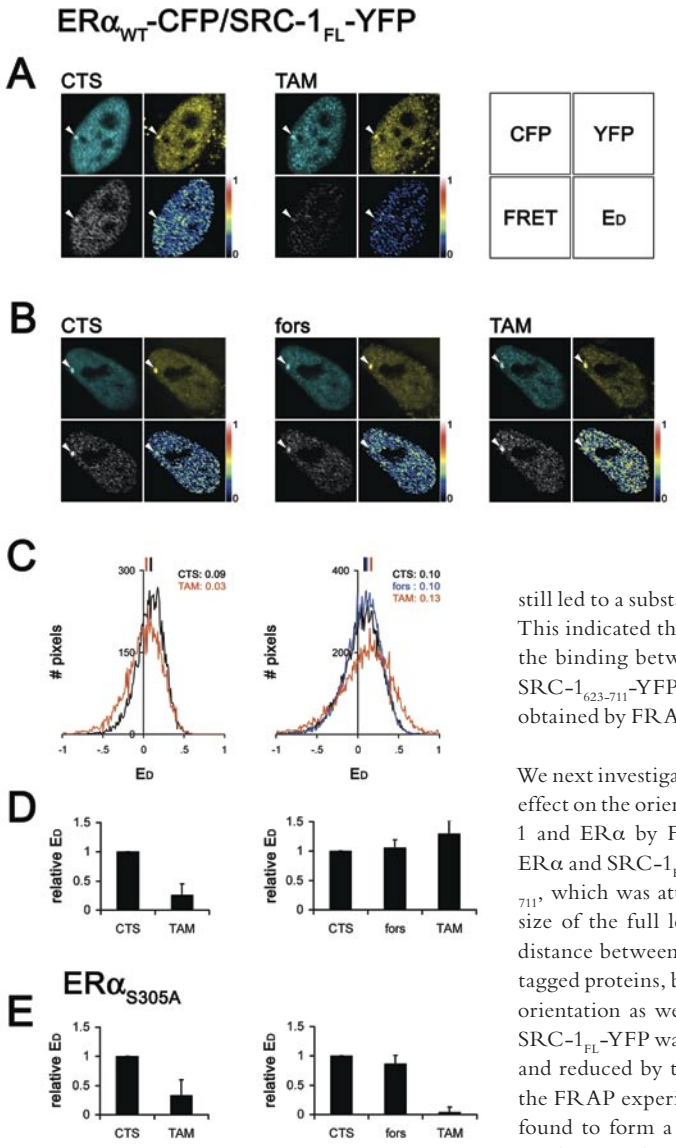

Figure 4. Orientation between ERα and SRC-1$_{FL}$ is altered by tamoxifen alone, but is stabilized by activation of PKA. FRET experiments were performed similar as described at Figure 3, now using SRC-1$_{FL}$-YFP and wild type ERα **(A–D)** or ERα$_{S305A}$ **(E)**. Arrows indicate the PRL DNA array.

and Methods, we observed that the average efficiency of 16% FRET between ERα-CFP and SRC-1$_{623-711}$-YFP under hormone depleted conditions was reduced to an average of 5% after addition of tamoxifen to this particular cell (**Figures 3C**) and others (**Figure 3D**). The contribution of noise leads to a population of pixels with negative E_D, as was shown before (24). PKA activation by forskolin under conditions of CTS did not significantly influence FRET efficiency between ERα and SRC-1$_{623-711}$ (**Figure 3B** and quantified in **Figure 3D**). Subsequent treatment with tamoxifen still led to a substantial reduction in FRET efficiency. This indicated that PKA activation did not influence the binding between the AF-2 domain of ERα and SRC-1$_{623-711}$-YFP, in good agreement with the results obtained by FRAP (**Figure 2**).

We next investigated whether PKA treatment had any effect on the orientation between the full length SRC-1 and ERα by FRET (**Figure 4**). FRET between ERα and SRC-1$_{FL}$ was less efficient as with SRC-1$_{623-711}$, which was attributed to the much larger protein size of the full length SRC-1 and hence the larger distance between the fluorophores at the ends of the tagged proteins, but may also be the result of different orientation as well. FRET between ERα-CFP and SRC-1$_{FL}$-YFP was present in hormone-deprived cells and reduced by tamoxifen (**Figure 4A**), confirming the FRAP experiments in which these proteins were found to form a complex under hormone-deprived conditions. The mean FRET efficiency of 9% in CTS was reduced to 3% when tamoxifen was added (**Figure 4C**). This is remarkable, since the M2H and FRAP experiments demonstrated that interaction between ERα and SRC-1$_{FL}$ was not altered in the presence of tamoxifen (**Figures 1** and **2**). We therefore concluded that a loss of FRET under conditions of tamoxifen indicated an altered orientation between ERα and SRC-1, where the C-termini of ERα and SRC-1$_{FL}$ that carry the fluorophores orient to each other differently in tamoxifen treated cells as under CTS or E2 conditions. We next investigated whether activation of PKA influenced this reorientation upon tamoxifen

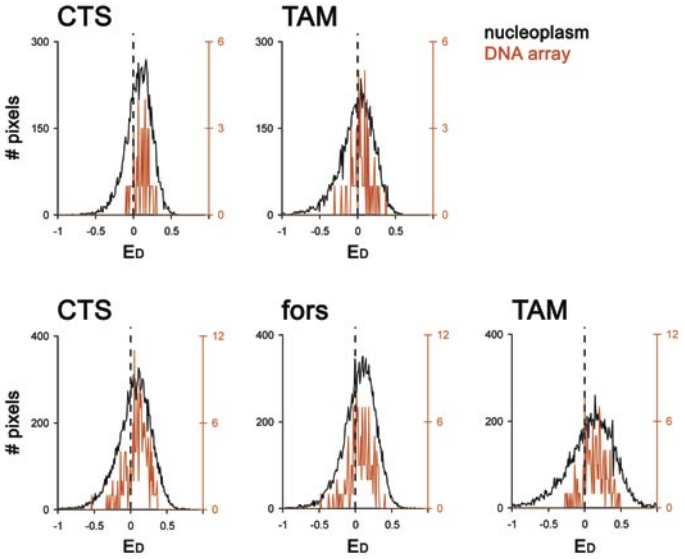

Figure 5. Interaction between ERα and SRC-1 is independent of DNA promoter content. FRET measurements were performed on PRL DNA array containing HeLa cells, transfected with $ERα_{wt}$-CFP and $SRC\text{-}1_{FL}$-YFP and cultured in CTS medium (see cases presented in Figures 4A and 4B). Where indicated, they were pretreated with 10 μM forskolin. Subsequently cells were treated with 1μM 4'OH-Tamoxifen for 2 hours and imaged. Donor FRET efficiency (E_D) was calculated as described in Experimental Procedures. The raw E_D values were related to the total amount of pixels and presented for both DNA array (in red) and entire nucleus (in black) for the cells shown in Figures 4A and 4B.

binding. PKA activation through forskolin by its own had no effect on FRET efficiency under hormone-deprived conditions (**Figure 4B**). However, subsequent treatment with tamoxifen now did not show a change in FRET efficiency as did occur when cells had not been pretreated with forskolin, indicating that the orientation between ERα and $SRC\text{-}1_{FL}$ is stabilized by activation of PKA. The $ERα_{S305A}$ mutant behaved in this assay similar as wild-type ERα under non-PKA stimulated conditions: forskolin treatment followed by tamoxifen still resulted in a loss of FRET (**Figure 4E** and **Supplemental Figure S1**). This strongly suggests that phosphorylation of S305 in wild-type ERα by PKA affects the orientation between ERα and full length SRC-1 (quantified in **Figures 4D** and **4E**). Taken together, these data show that the orientation between ERα-CFP and $SRC\text{-}1_{FL}$-YFP is altered by tamoxifen, and that this change in orientation does not occur when PKA phosphorylates S305 of ERα.

ERα interacts in the nucleus with SRC-1 irrespective of DNA promoter content.

The complex formation between ERα and SRC-1 is studied here by monitoring changes in FRET in the whole nucleus (**Figures 3** and **4**). Since the ERα transcription factor complex is effective on its responsive element in the DNA, we investigated whether the interaction between ERα and SRC-1 on a repeat of such a responsive element differs from that in the remainder of the nucleus. We used of HeLa cells containing a DNA array, consisting of a stably transfected 52 multimer of the prolactin promoter/enhancer (PRL) that contains an Estrogen Responsive Element (26). These cells contain a defined DNA structure in the nucleus on which ERα and cofactors are recruited. This permitted a clear distinction between defined ERE-associated ERα and SRC-1 events on the DNA array and in the remainder of the nucleus, as shown in **Figures 4A** and **B**, indicated by arrowheads. Now, we compared FRET efficiency on the DNA array with that throughout the remainder of the nucleus (**Figure 5**). The levels of ERα-CFP, SRC-1-YFP proteins and FRET events were significantly higher on the DNA array than outside, as shown in **Figure 4**. However, when FRET from these cells was related to the emission of the donor (E_D), making the readout fluorophore concentration independent, similar E_D values were obtained on the array as compared to the rest of the nucleus. Also, the effects on E_D of tamoxifen alone or in combination with forskolin on the DNA array were comparable with that off the array. (**Figure 5**). These results indicated that similar interactions between ERα-CFP and $SRC\text{-}1_{FL}$-YFP occurred on the DNA array as throughout the nucleus.

Phosphorylation of Serine-305 of ERα by PKA is responsible for recruitment of RNA polymerase II to the transcription complex and for enhanced transcription under tamoxifen conditions.

Genuine tamoxifen resistance would imply that the observed effect of PKA on the orientation between SRC-1 and ERα in the presence of tamoxifen would

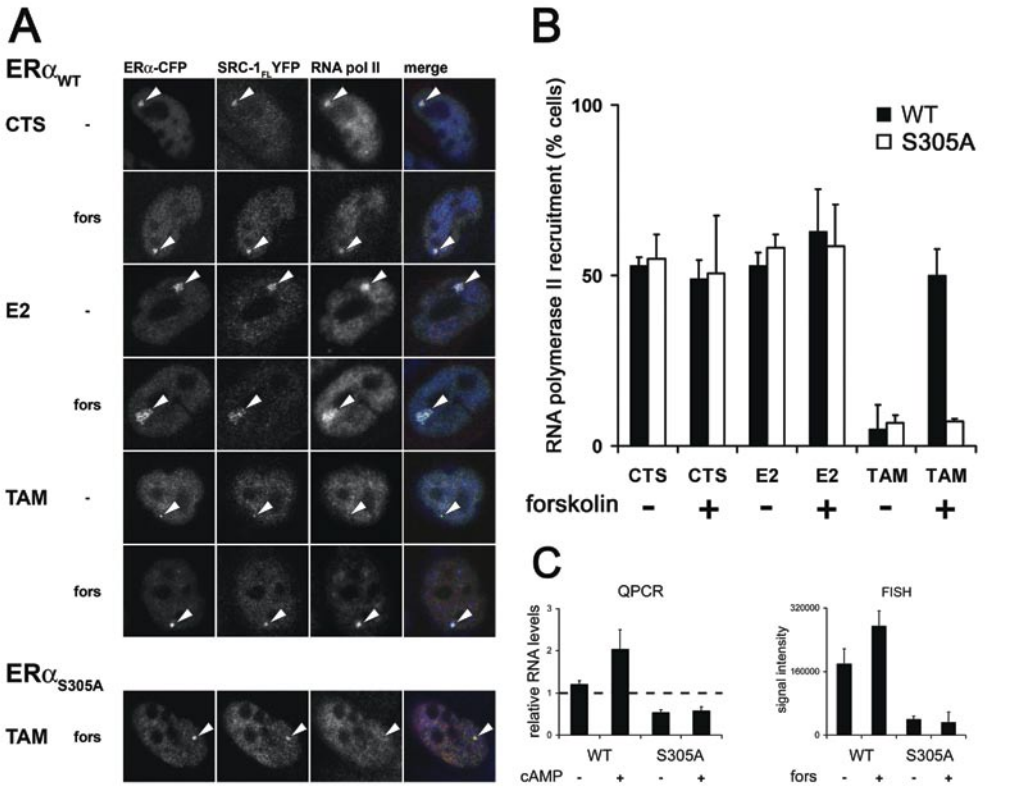

Figure 6. PKA-mediated phosphorylation of S305 of ERα is responsible for RNA polymerase II recruitment on the PRL DNA array and for enhanced transciption of a hormone-responsive reporter gene under conditions of tamoxifen. **(A)** Staining for RNA polymerase II was performed on PRL array containing HeLa cells that were transfected with $ER\alpha_{wt}$ or $ER\alpha_{S305A}$-CFP and $SRC1_{FL}$-YFP and cultured in medium containing only CTS. Where indicated, they were pretreated with 10 μM forskolin. Subsequently cells were treated with 1 μM E2 or 1μM 4'OH-Tamoxifen for 2 hours and then fixed and stained for RNA Polymerase II. Arrowheads indicate the PRL DNA array that was analyzed for RNA polymerase II staining. **(B)** Quantification of the RNA polymerase II signals on the PRL array under conditions as described in Figures 6A. Quantifications are from 3 independent experiments, n>40 cells each. Bars indicate standard deviations. **(C)** PKA-mediated phosphorylation of S305 on ERα enhances transcription of the PRL DNA array under conditions of tamoxifen. QPCR was performed on PRL DNA array containing HeLa cells, transfected with $ER\alpha_{wt}$-CFP or $ER\alpha_{S305A}$-CFP, which were treated with 1 μM Tamoxifen or left untreated (CTS). 16 hours prior to analysis 100 μM 8-Br-cAMP was added where indicated. mRNA levels were measured as described in Experimental Procedures, and related to CTS values which was set to 1. Bars indicate standard deviation from 2 independent experiments. RNA FISH of the hormone responsive DsRed gene behind the PRL DNA promoter in PRL DNA array HeLa cells transfected with $ER\alpha_{wt}$-CFP or $ER\alpha_{S305A}$-CFP. 2 hours prior to analysis cells were treated with forskolin where indicated and subsequently with 1μM 4'OH-Tamoxifen. Signal intensity of the FISH DsRed, as visualized by Alexa-546 is given. Bars indicate standard deviation from 2 independent experiments.

lead to tamoxifen-mediated transactivation of ERα with recruitment of RNA polymerase II to the ERα/SRC-1 complex as a hallmark. We examined the RNA polymerase II recruitment in the PRL DNA array containing cells that were transfected with ERα-CFP and $SRC\text{-}1_{FL}$-YFP. The transcriptional capacity of the complex is visualized by co-localization of the ERα/SRC-1 complex with RNA polymerase II on the PRL DNA array. Approximately half of the cells expressing both $ER\alpha_{wt}$-CFP and $SRC\text{-}1_{FL}$-YFP showed co-localization with RNA-polymerase II on the DNA array under conditions of CTS and E2 (**Figure 6A** and quantified in **6B**). The DNA array did show a more 'open' structure in the presence of E2 than with CTS. This open structure was previously reported to be associated with increased transcriptional activity

(26). Activation of PKA by forskolin did not affect co-localization of the three proteins under conditions of CTS and E2. Tamoxifen treated cells however, showed a more compact DNA array on which both ERα and SRC-1, but no RNA-polymerase II was recruited (**Figure 6A**). Strikingly, activation of PKA under tamoxifen conditions now resulted in recruitment of RNA polymerase II to the array, which was also slightly increased in size as compared to tamoxifen alone, indicating that PKA activation stabilized the orientation between ERα and SRC-1, which led to RNA polymerase II recruitment to the DNA array under tamoxifen conditions. Use of the S305A mutant of ERα did not result in RNA polymerase II recruitment and an increase of array-size under these conditions (**Figure 6A** and **Supplementary Figure S2**). This strongly suggests that RNA polymerase II recruitment and the observed increased DNA array size in tamoxifen treated cells are dependent on phosphorylation of S305 in ERα by PKA.

To investigate whether these events resulted in enhanced transcription, we measured transcription of the DsRed gene that was under control of the 52 multimer of the prolactin promoter/enhancer (26) by Quantitative RT-PCR and RNA FISH as described in Experimental Procedures (**Figure 6C**). PKA activation by cAMP led in tamoxifen treated cells transfected with $ERα_{wt}$ to a two-fold increase in transcription of the DsRed gene as compared with CTS conditions, whereas no such effect was observed in cells transfected with $ERα_{S305A}$. A similar PKA induced increase in DsRed transcripts was specifically associated with the PRL-DNA array as shown by RNA FISH in cells transfected with wild type ERα, but not in $ERα_{S305A}$ transfeceted cells, under tamoxifen conditions.

Our results indicated that tamoxifen resistance is due to a change in the orientation between ERα and SRC-1, which was dependent on PKA-mediated phosphorylation of S305. This reorientation between the C-termini of ERα and $SRC\text{-}1_{FL}$ was responsible for RNA polymerase II recruitment and for enhanced transcription.

Discussion

Regulated gene expression is achieved through the coordinated assembly of transcription factors, co-factors and the basal transcription machinery on promoters, and demands for a spatio-temporal coordination of interactions between these components. Traditional models of transcription tend to be static and depend on overall interactions measured by various kinds of binding assays, whereas the generation of the transcription factor complex requires a fine-tuned recruitment of components depending mainly on affinity of interfaces. The present study shows that the orientation of a tamoxifen-bound ERα transcription factor towards its coactivator SRC-1 is altered by PKA-mediated phosphorylation of S305 and that this reorientation is responsible for resistance to tamoxifen. This reorientation was observed by intermolecular FRET (**Figures 3** and **4**), whereas other methods, such as M2H and FRAP (**Figures 1** and **2**) showed no effect of PKA on overall binding between ERα and SRC-1 under tamoxifen conditions. This PKA-mediated reorientation of ERα leads to recruitment of RNA polymerase II (**Figure 6**), a hallmark for transcription, and to enhanced transcription from a hormone responsive reporter gene. Our study therefore, stresses the orientation of interfaces in the transcription complex to be crucial for efficacy of transcription and provides a mechanism for this kind of resistance to tamoxifen.

We found no difference in the FRET efficiency resulting from the interactions of ERα with SRC-1 on the DNA array or in the remainder of the nucleus (**Figure 5**). This indicates that a non-DNA bound ERα/SRC-1 complex is already formed throughout the nucleus, and that their relative orientations are unaltered by DNA binding. Similar conclusions have also been reached from FRAP studies on complexes of ERα (27) and of SRC-1 (15).

The PKA-mediated phosphorylation of S305 in the hinge region of ERα is responsible for a reorientation of the C-terminus of ERα under tamoxifen conditions, which we measured by FRET using C-terminally tagged ERα-CFP and SRC-1-YFP constructs. This reorientation alters the interaction between the C-termini of ERα and SRC-1 and depends on prolonged association via AF-1, since the AF-2 binding SRC-1 fragment did not interact with ERα under tamoxifen conditions, nor was interaction induced by PKA activation (**Figures 1, 2** and **3**). Our results thus support previous findings that AF-1 binding is a prerequisite for resistance to tamoxifen (28), whereas functional synergy between AF-1 and AF-2 is mandatory for recruitment of RNA polymerase II due to repositioning of AF-2 (9). Since the PKA effect on orientation occurs only in the presence of tamoxifen, and not under conditions of CTS, the AF-1 domain should be

regarded as a ligand dependent (in this case tamoxifen-dependent) transactivation domain after PKA activation. The AF-2 binding fragment of the SRC-1 construct that we used in this study as an AF-2 probe, encompasses aa 623-711 and contains two LXXLL motives that interact with the cofactor binding pocket in the AF-2 of the ERα-LBD (14). The results with this AF-2 probe in our M2H and FRAP experiments indicate that it binds to ERα under conditions of E2 and CTS, but not in the presence of tamoxifen. This binding was not influenced by activation of PKA. The full-length SRC-1_{FL}, however, binds to ERα under all three conditions when measured by M2H and FRAP and this binding is also not affected by PKA activation. Since SRC-1_{FL} binds to both AF-1 and AF-2 domains of ERα (9), and SRC-$1_{623-711}$ only to the AF-2 domain, our results indicate that the AF-1 functional core in ERα, which is positioned at the start of the B-domain (aa 39-45 of ERα) (9), is involved in the interaction between ERα and SRC-1 in tamoxifen treated cells. Interaction between this AF-1 helical core and SRC-1 has been reported to be required for ERα activity in the presence of tamoxifen (28) and is likely involved in the PKA-mediated reorientation between ERα and SRC-1 that we observed in our experiments under tamoxifen conditions. Since the outcome of our M2H and FRAP experiments that measure affinity between ERα and SRC-1_{FL} molecules, was not influenced by tamoxifen, whereas the outcome of the FRET experiments that measures distance and orientation between them, was affected by tamoxifen, we concluded that not the distance, but orientation between ERα and SRC-1_{FL} was altered by tamoxifen. This tamoxifen-associated alteration is prevented by phosphorylation of S305 of ERα by PKA, which stabilizes in the presence of tamoxifen the orientation towards SRC-1 as it appears under CTS conditions (**Figure 4**). This finding leads to a model where PKA-mediated phosphorylation of S305 results in a conformational state of ERα by which the orientation between ERα and SRC-1 is responsible for recruitment of RNA polymerase II under conditions of tamoxifen.

The effect of PKA on the conformation of ERα involves most likely the reorientation between the N- and C termini of ERα that we have observed by intramolecular FRET as has been reported previously (3). This intramolecular change was dependent on PKA-mediated phosphorylation of S305 and resulted in enhanced transactivation of ERα and tamoxifen dependent proliferation after PKA activation. The results of the present study strongly suggest that the change of ERα conformation upon PKA activation results in a stabile orientation between ERα and SRC-1, which renders the complex effective under tamoxifen conditions.

Binding of ERα to endogenous SRCs may have influenced the outcome of the experiments with overexpression of exogenous SRC-1. However, ERα interaction with SRC-3 is only enhanced by E2 stimulation, whereas SRC-1 was found to be present on PRL array structures also in the absence of any ligand (**Figure 3**) (26). Therefore overexpression of SRC-1 via transient transfection leads to most of the ERα being bound to SRC-1 in CTS. Besides to phosphorylation of direct target sites in ERα, phosphorylation of SRC-1 (29) and reduced association between ERα and transcriptional corepressors NCoR and SMRT (30) may contribute to PKA-mediated resistance to anti-estrogens. Since the PKA-induced reorientation, RNA Polymerase II recruitment and enhanced transcription were lost when the $ERα_{S305A}$ mutant was used in our experiments (**Figures 4E** and **Supplemental Figure S2**), we conclude that phosphorylation of S305 by PKA is the main target for PKA induced tamoxifen resistance that may still act in conjunction with phosphorylation of SRC-1 and NCoR/SMRT.

A fraction of the SRC-1 molecules (in our experiments 40%) appeared immobilized in the nucleus (Figure 2), whereas the size of this fraction was not affected by the various treatment conditions. This immobility is due to stabilization of SRC-1 on regions of ERα other than AF-2, since the major fraction of the truncated AF-2 binding mutant of SRC-$1_{623-711}$ was found to be freely diffusing. Only a minor fraction of the truncated SRC-$1_{623-711}$-YFP proteins was immobilized under in CTS, which was increased in the presence of E2, but all of it diffused freely under tamoxifen conditions. These results suggest that the AF-1 part of ERα is mainly responsible for stabilizing the binding to SRC-1, whereas the AF-2 part may loosely interact with SRC-1 and only becomes stabilized upon E2 binding. The reorientation of the C-terminus of tamoxifen-bound ERα towards SRC-1 that is induced by either PKA or PAK-1 mediated phosphorylation of S305 in the hinge region of ERα, provides a unique model for resistance to tamoxifen. It demonstrates that the effect of interacting agents can be nullified by activation of other signaling pathways, adding to the complexity of estrogen mediated transcriptional events and how to interfere with these. This mechanism also provides a framework for selection and development of agents

that are insensitive to these modifications on ERα and leads to identification of conditions for resistance to tamoxifen in breast cancer patients.

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