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SRA coactivation of estrogen receptor-α is phosphorylation-independent, and enhances 4-hydroxytamoxifen agonist activity

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Abstract

The ability of steroid receptor RNA activator (SRA), an AF-1 coactivator, to contribute to differences in estrogen receptor (ER)-α and ERβ transcriptional activity was tested. In transient transfections, SRA expression increased ERα- and ERβ-dependent gene expression. However, when the receptors' amino-terminal A/B regions were examined as GAL4 DNA binding domain fusions, SRA enhanced the activity of GAL-ABα but not GAL-ABβ. Exogenous SRA also enhanced AF-2 activity for both receptors, indicating that SRA effects are not limited to AF-1. Simultaneously mutating three phosphorylation sites within GAL-ABα domain only modestly reduced SRA coactivation of GAL-ABα, suggesting that phosphorylation does not play a major role in SRA function relative to this domain. SRA enhanced ERα activity stimulated by 4-hydroxytamoxifen, but was unable to convert this mixed antiestrogen to an ERβ agonist. Thus, SRA is an ERα AF-1-specific coactivator that enhances the agonist activity of tamoxifen-bound ERα and may contribute to tamoxifen resistance.

Keywords: Estrogen receptors; SRA; Tamoxifen; Phosphorylation; ERα; ERβ; AF-1; Coactivator

Estrogen receptor-α (ERα) and ERβ are members of a superfamily of ligand-regulated transcription factors that stimulate gene expression in response to 17β-estradiol (E2) and other estrogenic compounds. These receptors can be divided into structural/functional regions, designated A through F [1]. Both receptors encompass a centrally located DNA binding domain and two activation functions; the one located in the amino-terminal domain is referred to as activation function-1 (AF-1), while a second located in the carboxy-terminal ligand binding domain is named AF-2. The AF-1 domain is constitutively active, but functions in a cell- and promoter-dependent manner [2,3]. In contrast, the AF-2 domain contributes to the transcriptional activity of both ERα and ERβ in a ligand-dependent fashion.

Estrogen receptors enhance gene expression by recruiting coactivator proteins with the ability to modify the local chromatin structure to target gene promoters [4–6]. Multiple estrogen receptor coactivators have been identified and characterized, including members of the p160 steroid receptor coactivator-1 (SRC-1) family. Recently, a coactivator was identified and termed steroid receptor RNA activator (SRA) because of its ability to function as an RNA transcript [7]. It was further shown to activate progesterone receptor-B, glucocorticoid receptor (GR)-, and ERα-, but not ERβ-dependent transcription via their respective AF-1 domains [7–9].

Interestingly, SRA expression was found to be altered in human breast cancer tissue samples, suggesting that this coactivator might be involved in mediating cell

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proliferation [10,11]. Because mammary gland proliferation is strongly dependent on estrogens, this also suggested a possible link between SRA and ERs in vivo. Thus, a better understanding of how each ER subtype relates to SRA as well as other coactivators could improve strategies for treating breast cancer.

Tamoxifen stimulation of classical ER-mediated gene expression is dependent on the receptor's AF-1 domain, and it has been demonstrated that this antiestrogen blocks AF-2, but not AF-1, function [12,13]. The ability of 4-hydroxytamoxifen (4HT) to act as a partial agonist of ERα, but not ERβ on ERE-containing target genes is therefore thought to be a reflection of the distinct activities of the ERα and ERβ AF-1 domains [14–16]. In the present study, SRA acts as a coactivator for both ERα and ERβ, but stimulates only the AF-1 activity of ERα. Consistent with this, SRA coactivates the activity of ERα in the presence of the partial agonist, 4HT. In contrast, the coactivation of ERβ by SRA is mediated via this receptor's AF-2 domain suggesting that SRA can enhance AF-1 and/or AF-2 function depending on the ER subtype and promote the agonist activity of an antiestrogen used to treat breast cancer.

Materials and methods

Chemicals. 17β-Estradiol (E_2) was obtained from Sigma Chemical (St. Louis, MO). The antiestrogens, ICI 182,780 and 4HT, were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively.

Plasmid DNAs. The mammalian expression plasmids for human ERα ($pCMV_5$-hERα [17]; pCR3.1-hERα [18], or $pRST_7$-hERα [3]), the full-length ($pCXN_2$-hERβ [19]), and the AF-2 mutants of hERα ($pRST_7$-hER-3x and $pRST_7$-hER-TAF2-3x [3]) were described previously as were the synthetic target genes, pERE-E1b-Luc [18] and pC3-Luc [3]. The pG5-Luc target gene, which contains five binding sites for the GAL4 DNA binding domain (DBD) fused to the luciferase gene, was obtained from Promega (Madison, WI). The mammalian expression vector for SRA (pSCT-SRA) and its corresponding parent vector (pSCT) have been used previously [7], as has the pCR3.1-SRC-1e plasmid for SRC-1e [20]. The mammalian expression plasmids for the GAL4 DNA binding domain linked to the A/B domain of ERα (GAL-ABα) as well as the corresponding constructs containing serine to alanine (S118A, S104/106A, and S104,106,118A) mutations have also been described [20].

The constructs for hERα-179C (pCR3.1-hERα-179C) and hERβ-143C (pCR3.1-hERβ-143C) were made by PCR using the primers 5′-ACCATGGCCAAGGAGACTCGCTACTGT-3′ and 5′-CTCTCAG ACTGTGGCAGGGAAACC-3′ to amplify the segment of $pCMV_5$-hERα encoding amino acids 179–595 and the primers 5′-ACCA TGAAGAGGGATGCTCACTTCTGC-3′ and 5′-GCGTCACTGAG ACTGTGGGTTCTG-3′ to PCR amplify the segment of $pCXN_2$-hERβ cDNA encoding residues 143–530, respectively. Each of the resulting PCR fragments was cloned into the pCR3.1 expression plasmid using the TA cloning kit. All PCR products were verified by sequence analysis to ensure that errors did not occur during their synthesis.

The Gal-A/Bβ chimera was constructed as follows. First, PCR amplification was performed using the primers 5′-GGGCCGGG ATCCCGATGGATATAAAAAACTCACCATCT-3′ and 5′-CCCCG GGATCCTTAAGCATCCCTCTTTGAACCTGG-3′ to amplify the region of the human ERβ ($pCXN_2$-hERβ) cDNA encoding amino acids 1–146. Each of these primers possesses a *Bam*HI restriction site (underlined). The resulting cDNA fragment was subcloned into the pCR3.1 TA cloning vector, and subsequently the *Bam*HI cDNA fragment was removed by restriction digest and subcloned into the pBind vector (Promega) in-frame with and downstream of the coding sequence for the GAL4 DNA binding domain. The resulting vector was sequenced to ensure that no errors were introduced during plasmid construction.

Cell culture and transfections. Human cervical carcinoma (HeLa) and hepatocellular carcinoma (HepG2) cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty four hours prior to transfections, cells were plated in six-well culture dishes at a density of 3×10^5 (HeLa) or 8×10^5 (HepG2) cells per well in phenol red-free DMEM with 5% charcoal-stripped fetal bovine serum (sFBS). DNA was introduced into cells in the indicated amounts using Lipofectin or Lipofectamine (Invitrogen, Carlsbad, CA). Eight hours later, serum-free media were replaced with phenol red-free DMEM supplemented with 5% sFBS, and 12–20 h thereafter, cells were treated with the indicated amounts of various hormones. After hormone treatment, cells were harvested and extracts were assayed for luciferase activity using the Luciferase Assay Systems kit (Promega) and a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Relative luciferase units were normalized to total cellular protein, as determined by Bio-Rad Protein Assay. Experiments were done in duplicate and values represent means ± SEM of at least three individual experiments.

Western blot analysis. To determine ER expression levels for the GAL-ABα and its corresponding mutant constructs, cells were transfected as above, and harvested and lysed as described previously [21]. Cell extracts were resolved by 7.5% SDS–PAGE and subjected to Western blot analysis using an antibody to the GAL DBD (Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish peroxidase-conjugated, anti-mouse antibody. Blots were visualized using enhanced chemiluminescence reagents as recommended by the manufacturer (Amersham–Pharmacia Biotech, Piscataway, NJ).

Results

To assess the ability of SRA to coactivate ERα and ERβ transcriptional activity, SRA was overexpressed in cells transfected with expression vectors for full-length ERα or ERβ and the estrogen receptor-dependent synthetic target gene, ERE-E1b-Luc, which consists of a consensus estrogen response element linked upstream of a TATA box and luciferase reporter gene. In response to estradiol, both ERα and ERβ activated luciferase expression (Fig. 1), and as expected, full-length ERβ was ~50% less active in comparison to ERα (data not shown). However, the ability of SRA overexpression to enhance receptor-dependent target gene expression did not vary; both ERα and ERβ activities were stimulated approximately threefold.

The mixed antiestrogen, 4HT, is a relatively good agonist in HepG2 cells [3] where ERα activity is highly dependent upon AF-1 activity. One hundred nanomolar 4HT was able to stimulate the transcriptional activity of ERα in HepG2 cells as well as 10 nM estradiol (Fig. 2A). Furthermore, SRA overexpression stimulated the transcriptional activity of ERα in the presence of

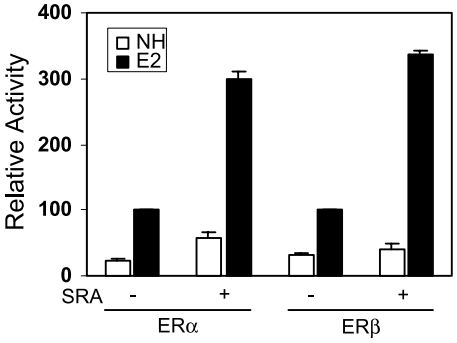

Fig. 1. Coactivation of human ERα and ERβ by SRA overexpression. HeLa cells were cotransfected with 40 ng pCMV$_5$-hERα or pCXN$_2$-hERβ, and 1500 ng ERE-E1b-Luc in the presence or absence of 1000 ng pSCT-SRA and treated with ethanol (NH) or 1 nM E2. Values are the average ± SEM of three independent experiments standardized to the luciferase data obtained in the presence of E2 and absence of transfected SRA expression vectors.

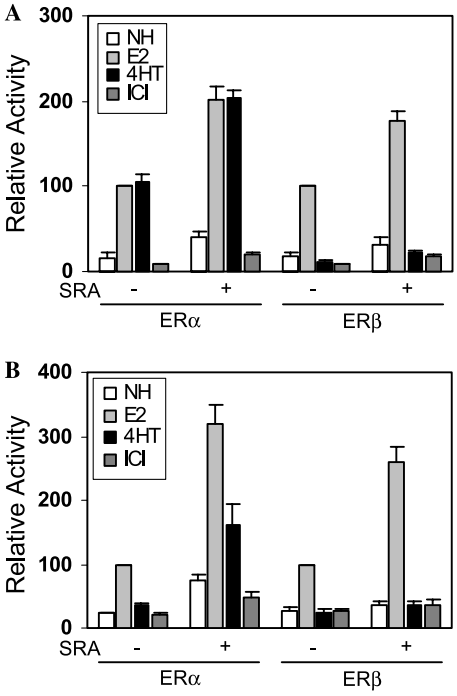

Fig. 2. SRA coactivates ERα transcriptional activity in the presence of mixed, but not pure antiestrogens in HepG2 and HeLa cells. (A) HepG2 cells were cotransfected with 50 ng pCMV$_5$-hERα or pCXN$_2$-hERβ, and 1000 ng pC3-Luc in the presence or absence of 1000 ng pSCT-SRA. (B) HeLa cells were cotransfected with 40 ng pCMV$_5$-hERα or pCXN$_2$-hERβ, and 1500 ng ERE-E1b-Luc in the presence or absence of 1000 ng pSCT-SRA. Cells were treated with ethanol (NH), 10 (A) or 1 (B) nM E2, 100 nM 4HT or 100 nM ICI 182,780. Values are the average ± SEM of three (A) or 4–7 (B) independent experiments standardized to the luciferase data obtained in the presence of estrogen and absence of transfected SRA expression vectors.

4HT to the same extent as estradiol. When the experiment was performed with ERβ, the luciferase activity induced by E2 was only ~40% as great as that stimulated by ERα (data not shown), and consistent with ERβ's refractoriness to mixed antiestrogens [16,22,23], no activation of target gene expression was detected in 4HT-treated cells.

The above experiment was repeated in HeLa cells in order to determine whether SRA overexpression could promote the agonist potential of 4HT in a cell environment in which this compound is typically an ER antagonist. As shown in Fig. 2B, in the absence of exogenous SRA, 4HT agonist activity is very weak. However, overexpression of SRA increased the ability of 4HT to activate gene expression in comparison to estradiol (~4-fold versus 3-fold, respectively), suggesting that ERα liganded with 4HT was able to functionally interact with SRA. Consistent with the HeLa cell results, 4HT alone was unable to stimulate the transcriptional activity of ERβ in this system and SRA overexpression was unable to rescue the inability of 4HT to stimulate ERβ-dependent gene expression (Fig. 2B).

The ability of the pure antiestrogen ICI 182,780 to activate transcription in the absence and presence of exogenous SRA also was assessed (Fig. 2). This antiestrogen inhibits ERα and ERβ transcriptional activity in most contexts, and in HeLa or HepG2 cells it was unable to stimulate the activity of either ERα or ERβ whether or not SRA was cotransfected. These data indicate that the ability of SRA to stimulate the transcriptional activity of ERs liganded with antiestrogens depends on the nature of the antiestrogen [partial (4HT) versus pure (ICI 182,780)] and the subtype of estrogen receptor (α versus β).

In order to determine if SRA could coactivate the transcriptional activity of just the AF-1 domains of each receptor subtype, expression vectors encoding fusions of the GAL4 DBD to either the A/B domain of ERα (GAL-ABα) or ERβ (GAL-ABβ) were made. These were cotransfected into HeLa cells with a reporter gene containing five GAL4 DNA binding sites (pG5-Luc). As shown in Fig. 3A, the A/Bα domain in the absence of exogenous coactivator has considerable transcriptional activity in comparison to either the GAL4 DBD alone or the GAL-ABβ chimera. Overexpression of SRA stimulated the ability of the GAL-ABα activity nearly threefold but had no effect on the weak transcriptional activity of GAL-A/Bβ or basal activity of GAL4 DBD alone. Coactivation of an AF-2 mutant of ERα (ERα-3x), which is severely compromised in its ability to interact with the SRC-1 and GRIP1 coactivators via its AF-2 domain [24], demonstrated further that SRA coactivates the ERα AF-1 domain in the context of the full-length receptor (Fig. 3B). However, when the AF-1 domain was deleted in addition to the AF-2 point mutations to yield the ERα-TAF2-3x mutant, no hormone-induced transcriptional activity was observed, and SRA overexpression was unable to substantially increase luciferase gene expression. Taken together, these data demonstrate

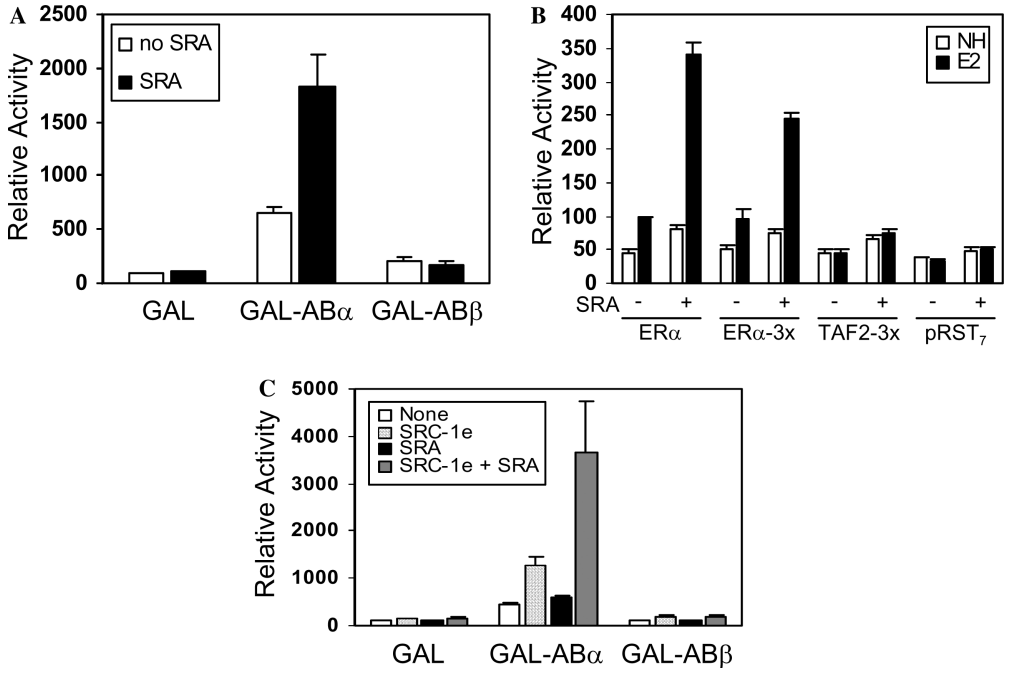

Fig. 3. Coactivation of the ERα AF-1 domain by SRA. (A) SRA coactivation of GAL-ABα and GAL-ABβ chimeric proteins by SRA. HeLa cells were cotransfected with 100 ng pBIND (GAL), pBIND-ABα (GAL-ABα) or pBIND-ABβ (GAL-ABβ) and 1000 ng pG5-Luc in the presence or absence of 1000 ng pSCT-SRA. Values are the average ± SEM of four independent experiments standardized to the luciferase data obtained for the GAL4 DNA binding domain in the absence of transfected SRA expression vectors. (B) SRA coactivation of an AF-2 mutant of ERα. HeLa cells were cotransfected with 250 ng of the $pRST_7$ expression vector for wild type, 3x or TAF2-3x forms of ERα or parent vector alone along with 1500 ng ERE-E1b-Luc in the presence or absence of 1000 ng SCT-SRA and treated with ethanol (NH) or 1 nM E2. Values are the average ± SEM of five independent experiments standardized to the luciferase data obtained for wild type ERα in the presence of estrogen and absence of transfected SRA expression vectors. (C) Coactivation of the ERα AF-1 domain by simultaneous coexpression of SRA and SRC-1e. HeLa cells were cotransfected with 40 ng of the expression vectors for GAL, GAL-ABα or GAL-ABβ and 1500 ng pG5-Luc in the presence or absence of 1000 ng pSCT-SRA and 1000 ng pCR3.1-SRC-1e. Values are the average ± SEM of five independent experiments standardized to the luciferase data obtained for GAL4 in the absence of transfected expression vectors for coactivators.

that SRA is an ERα-specific, AF-1 coactivator in the context of the A/B domain alone or the holoreceptor.

SRA has been detected in complexes with SRC-1 [7], and expression vectors for these coactivators were cotransfected to determine if SRC-1 overexpression could facilitate SRA coactivation of ERβ's AF-1 activity. As shown in Fig. 3C, coexpression of SRA and SRC-1 increased the transcriptional activity of the ERα A/B domain to an extent greater than either coactivator alone. In contrast, only SRC-1 was able to stimulate the transcriptional activity of the ERβ A/B domain, and the addition of exogenous SRA did not modulate the magnitude of this response. This finding suggests that SRA functional and/or physical interactions with the A/B domains of type I nuclear receptors are not simply the result of SRA interactions with SRC-1, and highlights the specificity of SRA for the ERα AF-1 domain.

Phosphorylation of the A/B domain is important for maximal AF-1 activity and to assess the role of this post-translational modification in mediating SRA coactivation, mutations resulting in the substitution of non-phosphorylatable alanines for serines at positions 104, 106, and 118 were introduced into the GAL-ABα expression plasmid. In the absence of SRA, mutating each serine individually or in combination reduced transcriptional activity (up to ~47% for the triple mutant, S104/106/118A) in comparison to the wild type construct (Fig. 4A). Western blot analysis demonstrated similar expression levels for the wild type and mutant receptors (Fig. 4B). Overexpression of SRA enhanced the transcriptional activity of each of the A/B domains, regardless of phosphorylation site mutations; the fold coactivation of each construct by SRA is shown in the inset, and only for the S104/106/118A mutant was coactivation by SRA significantly attenuated (Fig. 4A). Thus, the ability of the A/B domain to be coactivated by SRA is not strictly dependent on ERα phosphorylation, although it is clear that blocking receptor phosphorylation reduces the ability of SRA to stimulate AF-1 activity.

In order to determine if regions other than the A/B domain could support coactivation by SRA, ERs lacking their entire A/B domain (ERα-179C and ERβ-143C) were generated (Fig. 5). For neither receptor did deletion of the AF-1 region block SRA coactivation of receptor-dependent gene expression. Taken together, these data suggest that SRA, while a coactivator of

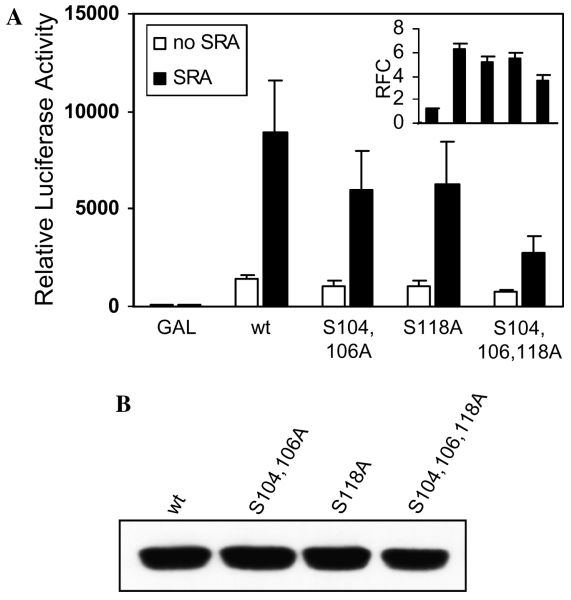

Fig. 4. SRA coactivation of GAL-AB-α wild type and its corresponding phosphomutant constructs. (A) HeLa cells were cotransfected with 100 ng pBind (GAL), wild type GAL-ABα (wt), or the indicated GAL-ABα phosphomutants along with 750 ng pG5-Luc in the presence or absence of SRA expression vector. Values are the average ± SEM of three experiments standardized to the luciferase data obtained for GAL alone in the absence of SRA. Relative fold coactivation (RFC) represents luciferase activity for each construct in the presence of SRA divided by the activity in the absence of SRA (inset). (B) Western blot analysis of the GAL-ABα wild type and its corresponding phosphomutants protein expression. Protein was detected using an antibody directed against the GAL4 DNA binding domain and is representative of two experiments.

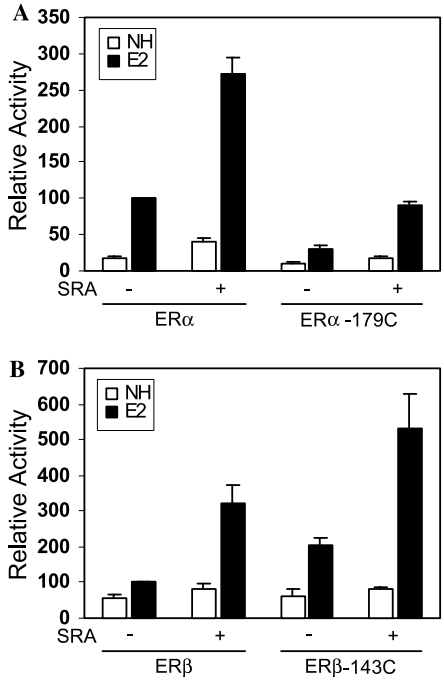

Fig. 5. Activation of amino-terminal deletion mutants of ERα and ERβ by SRA in HeLa cells. (A) Forty nanograms of the $pCMV_5$ expression vectors for ERα and ERα-179C ($n = 3$), or (B) pCR3.1 expression vectors for wild type ERβ and ERβ-143C ($n = 4$) were cotransfected with 1500 ng ERE-E1b-Luc in the presence or absence of 1000 ng pSCT-SRA and treated with ethanol (NH) or 1 nM E2. Values are the average ± SEM of the indicated number of independent experiments standardized to the luciferase data obtained for wild type receptor in the presence of estrogen and absence of transfected SRA expression vectors.

the AF-1 domain of only ERα, also possesses the ability to coactivate transcription via the ligand and/or DNA binding domains of both ER subtypes.

Discussion

The identification of a second receptor for estrogens, ERβ, has substantially increased the potential complexity of estrogen actions in vivo, and much effort is underway to resolve the mechanistic and physiological consequences of this second ER subtype. In view of the divergent amino-terminal sequences of ERα and ERβ, and the reported ability of SRA to activate the AF-1 domain of steroid receptors [7], as well as to better understand the mechanistic basis for differences in the ability of ERα and ERβ to stimulate gene expression in the presence of estrogens and antiestrogens, we conducted a series of experiments to characterize the ability of these receptors to functionally interact with the RNA coactivator, SRA.

Exogenous SRA increased levels of both ERα- and ERβ-dependent gene expression regardless of cell type (HeLa versus HepG2) or promoter (ERE-E1b-Luc versus pC3-Luc) tested, suggesting that the relative difference in these two receptor subtypes to activate target gene transcription was not due to an inability of SRA to functionally interact with ERβ. In addition, SRA and SRC-1 appear to work together, since activation of target gene expression is greater following transfection of expression vectors for both coactivators than would be anticipated from either coactivator alone, and this is consistent with the presence of SRA in SRC-1, but not p300 containing complexes isolated from T47D cells [7]. At present, the mechanism(s) by which SRA alters ER-dependent target gene expression is unclear. In general, there is an increasing appreciation for the potential roles that RNA plays in regulating transcription, including even functions more generally associated with proteins. For example, SRA has been shown to interact with and recruit a novel protein called SHARP to an ERα-dependent promoter [25], suggesting that this RNA may regulate gene expression in *trans* by assuming a structural role and recruiting proteins to promoters.

Interestingly, when tethered to the GAL4 DBD, the A/B domain of ERα was significantly more active than the corresponding region of ERβ, and only the ERα AF-1 domain was coactivated by SRA; this confirms ear-

lier work [8,9,26]. The basis for the differences in ERα and ERβ A/B domain activities is presumably related to the relative ability of these regions to physically and/or functionally interact with coactivators. It had been shown previously that this region of both receptors can interact with SRC-1, suggesting that the relative inactivity of the ERβ A/B domain is not due to a failure of this region to bind p160 coactivators [27,28]. Although it is not clear whether SRA binds directly to the A/B domain of nuclear receptors, there is a distinct difference in the ability of SRA to functionally interact with this region of ERα in comparison to ERβ and this likely contributes to the relatively poor transcriptional activity of the ERβ A/B domain. A synergistic response was observed only when SRA and SRC-1 were coexpressed in the GAL-ABα, but not GAL-ABβ transfected cells, further emphasizing the inability of SRA to functionally interact with the A/B domain of ERβ. This result also argues that the functional interaction of SRA with the ERα AF-1 is not simply the result of its recruitment to that region via SRC-1, and suggests that SRA either interacts directly with the A/B domain of ERα but not ERβ, or that it interacts with and/or requires other factors that selectively associate with the A/B domain of ERα.

The ability of SRA to coactivate the AF-1 domain of ERα in HeLa cells is not strictly dependent on phosphorylation of the three serine residues that were examined since SRA was still able to coactivate the individual GAL-ABα phosphomutant constructs to the same extent as the corresponding wild type receptor; only the serine 104/106/118 triple mutant was modestly compromised in its ability to be coactivated by SRA. This result contrasts with a previous report, which indicated that mutation of serine^{118} or inhibition of the MAP kinase signaling pathway responsible for serine^{118} phosphorylation completely abolished SRA coactivation of the ERα AF-1 domain [8]. Notably, different receptor constructs, cell types, and reporters were used in these two studies and this may explain the contrasting observations. Moreover, this suggests that SRA coactivation of ER may be cell type and/or promoter dependent.

The ability of SRA to coactivate mutant forms of ERα and ERβ lacking their entire A/B domains indicates that SRA, like the p160 family of coactivators [27,29], can stimulate transcriptional activity through the amino- and carboxy-terminal portions of the receptor. The demonstration that SRA could be detected in complexes immunoprecipitated with an androgen receptor antibody from *Xenopus* oocytes expressing an AF-1 deletion mutant of androgen receptor only when SRC-1 was coexpressed [7] suggests that SRA is able to physically interact with the carboxy-terminal portion of a nuclear receptor through interactions with SRC-1, and it is possible that SRA enhanced the transcriptional activity of the AF-2 domains of ERα and ERβ through a similar mechanism. Alternatively, a recent report demonstrated that SRA could interact directly with the thyroid hormone receptor via an RNA binding domain located in this receptor's hinge region [30].

Tamoxifen is a member of a class of drugs referred to as selective estrogen receptor modulators (SERMs), that can manifest agonist or antagonist activity depending on the cell, promoter, and estrogen receptor subtype [31]. Tamoxifen antagonizes AF-2 activity through blocking coactivator interaction(s) with this region of the receptor [32], but it does not inhibit the AF-1 domain of ERα, and deletion experiments indicate that the A/B region is required for tamoxifen-stimulated gene expression [13,33]. Thus, this SERM's agonistic properties are believed to be dependent on cell-specific coactivator interactions with ERα's AF-1 domain [31,34]. The inability of tamoxifen to stimulate ERβ transcriptional activity on ERE-containing target genes implies an AF-1 deficiency relative to ERα, and this is consistent with the lack of SRA coactivation of the ERβ AF-1 domain [14,26]. Nonetheless, the ability of SRA to stimulate the activity of the ERα AF-1 domain indicates that this coregulator may contribute to the tissue-specific ability of ERα to respond to SERMs in an agonist manner, including instances in which tumor cells acquire resistance to tamoxifen treatment. In support of this, elevated expression of an SRA variant has been strongly correlated with increased mammary cell proliferation and breast tumors [11,35], raising the intriguing possibility that changes in SRA expression may have implications for the sensitivity of breast tumors to estrogenic growth stimuli as well as antiestrogen (e.g., tamoxifen) therapy.

Acknowledgments

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