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Ligand-independent coactivation of ERα AF-1 by steroid receptor RNA activator (SRA) via MAPK activation☆

Geneviève Deblois[a,b], Vincent Giguère[a,*]

[a] *Molecular Oncology Group, McGill University Health Center, Room H5-21, 687 Pine Avenue West, Montréal, Que., Canada H3A 1A1*
[b] *Departments of Biochemistry, Medicine and Oncology, Faculty of Medicine, McGill University, Montréal, Que., Canada*

Abstract

Nuclear receptor coactivators are factors that enhance the transcriptional activity of the receptor. Coactivators usually work in ligand-independent and/or dependent manners by interacting with activation function-1 (AF-1) and AF-2 of the receptor, respectively. The recently characterized steroid receptor RNA activator (SRA) was cloned as an AF-1-dependent coactivator and shown to enhance the transcriptional activity of selected steroid receptors. In this work, we describe the effect of SRA on the activity of the two estrogen receptor (ER) isoforms, ERα and ERβ. We show that SRA potentiates the estrogen-induced transcriptional activity of both ERα and ERβ. We demonstrate that the transcriptional activity of ERα can be enhanced by SRA in a ligand-independent manner through the AF-1 domain. However, this AF-1-dependent effect of SRA is not observed on ERβ, denoting the ability of SRA to mediate differential activation of ERα and ERβ. The presence of an intact serine residue at position 118 (S^{118}) in ERα AF-1 is required for coactivation of ERα by SRA. We also show that activation of the mitogen activated protein kinase (MAPK) induces ligand-independent coactivation of ERα by SRA, a mechanism that is independent of the AF-2. Finally, SRA is unable to rescue the loss of activity of the S^{118} ERα mutant in response to H–Ras^{V12}, suggesting that phosphorylation of S^{118} by MAPK participates in the ligand-independent effect of SRA on ERα.

Keywords: Estrogen receptor; Coactivator; Transcription

1. Introduction

The developmental and physiological effects of 17β-estradiol (E_2) are now known to be mediated by two receptors, ERα [NR3A1] and ERβ [NR3A2], that are members of the nuclear receptor superfamily of transcription factors [1–5]. Despite the fact that both ERs share similar structural and functional properties, it is now clear that they possess individual characteristics conferring to them specific functions and complementary roles [6–8].

Both ERs mediate their transcriptional activity through the presence of two activation function domains that serve as docking sites for coactivators and general transcription factors (GTFs). Activation function-1 (AF-1) is located within the highly variable N-terminal region of the receptor, while AF-2 is located in the well conserved C-terminal ligand binding domain (LBD). AF-1 works in a ligand-independent manner and its activity can be modulated by phosphorylation events [9]. On the other hand, AF-2 works in a ligand-dependent manner: binding of the ligand to ER LBD induces repositioning of helix 12 on the surface of the LBD and allows it to make contact with the coactivators [10]. The concerted action of both AFs is usually required for maximal response to E_2 [11–13].

The nuclear receptor coactivators can modulate the transcriptional activity of ERs via a number of mechanisms. Among various functions, coactivators can perform or recruit enzymatic activities such as histone acetyl transferase and serve as bridging factors between ER, GTFs and cointegrators [14]. Most coactivators are thought to be part of large complexes that are recruited to the promoter and actively participate in transcriptional initiation. One of the most studied classes of coactivators is the p160/SRC family that includes SRC-1 (NCoA1), SRC-2 (NCoA2/GRIP-1/TIF2) and SRC-3 (NCoA3/pCIP/ACTR/AIB1/RAC3) [14]. The members of this family of coactivators interact ligand-dependently with ER AF-2 via their LXXLL motifs termed NR boxes (or LXDs) [15–17]. Some coactivators have also been shown to be recruited in a ligand-independent manner to the AF-1 of the ERs [13,18,19]. In particular, SRC-1 was shown to be recruited to and activate ERβ AF-1 upon MAPK-induced phosphorylation of serine residues 106 (S^{106}) and 124 (S^{124})

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* Corresponding author. Tel.: +1-514-843-1406; fax: +1-514-843-1478.
E-mail address: vincent.giguere@mcgill.ca (V. Giguère).

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[19]. Phosphorylation of serine residues in ERα AF-1 have also been associated with ligand-independent activity of this receptor [20]. However, phosphorylation-induced recruitment of p160/SRC members to the ERα AF-1 is less characterized. One of the major targets for phosphorylation in ERα AF-1 is serine residue 118 (S^{118}) [21]. Its phosphorylation is mediated by MAPK activation in the absence of ligand and induces AF-1-dependent ERα activity [20,22]. S^{118} is also phosphorylated upon ligand binding. E_2 binding to the LBD recruits TFIIH and its associated kinase Cdk7 to the AF-2 of the receptor, which in turn phosphorylates S^{118}. This event is required to mediate full activation of the receptor [23]. The integrity of S^{118} and its phosphorylation have proven to be essential both in the ligand-dependent and independent activation of ERα, suggesting that it might serve an important role in mediating the activation of both the AF-1 and AF-2 of the receptor [24]. It has also been suggested that phosphorylation of S^{118} recruits p68 RNA helicase (p68), an ERα AF-1 coactivator [25]. However, no direct recruitment of p160/SRC members to phosphorylated S^{118} has been reported.

The steroid RNA activator (SRA) is a recently characterized coactivator that has been shown to mediate its action via the AF-1 of steroid receptors [26]. The particularity of SRA resides in that it was shown to act as an RNA molecule [26,27]. SRA was demonstrated to be part of a larger complex containing SRC-1 [26], and to associate with the AF-1 specific p72 coactivator [28] and the corepressor Sharp [29], suggesting that SRA might play a central role in the activation of steroid receptors. The levels of SRA mRNA expression have been shown to vary during breast cancer progression [30,31]. Its coactivation potential was studied in more details with the progesterone receptor (PR), and it was shown that SRA could mediate coactivation for a PR mutant that lacks the LBD. Ligand-dependent SRA coactivation of a PR mutant lacking the AF-1 was also reported and the full-length PR was shown to be coactivated by SRA only in a ligand-dependent manner [26].

In this study, we investigated the coactivation of ERα and ERβ by SRA and found that SRA activation of ERα AF-1 is ligand-independent, an effect that is not observed for ERβ. Furthermore, we report that SRA can enhance the MAPK-mediated activation of ERα AF-1 in the absence of ligand, and this effect requires the integrity of S^{118} in ERα.

2. Materials and methods

2.1. Plasmid construct and reagents

p-CMX, p-CMX-hERα, p-CMX-hERβ, CMV-βGal, TKLuc and 2C-vERE-TKLuc were described previously [5]. All the ERα and mutants were constructed by PCR site-directed mutagenesis using *Pfu* polymerase (Stratagene) and the smallest possible fragment containing the mutation(s) was sequenced, cut out and reinserted into the template plasmid in order to reduce the risk of undesired mutation. The integrity of all plasmids described was verified by DNA sequencing. SRC-1 was a gift from Dr. Joe Torchia, University of Western Ontario, London, Ont., Canada. H-ras^{V12} was a generous gift from Dr. Morag Park, McGill University. SRA was a generous gift from Bert W. O'Malley, Department of Cell Biology and Biochemistry, Baylor College of Medicine, Houston, TX, USA. E_2 was obtained from Sigma Chemical Co. (St. Louis, MO). The specific MAPK inhibitor PD98059 was obtained from New England Biolab.

2.2. Cell culture and transfections

COS-1, HeLa and 293T cell lines were obtained from the American Type Culture Collection (ATCC). Cells were routinely cultured in phenol-red free Dulbeco's Minimal Essential Medium (DMEM) containing penicillin (25 U/ml), streptomycin (25 U/ml) and 10% fetal calf serum (FCS) or alternatively 10% steroid-deprived FCS, at 37 °C with 5% CO_2. Twenty-four hours prior to transfection, the cells were split and seeded into 12 well dishes. At this stage, the media containing 10% of complete FCS was replaced by phenol-red free DMEM supplemented with antibiotics and 10% charcoal-dextran treated FCS (steroid deprived). Cells were transfected using the Fugene-lipofectin method (Roche Diagnostic). Typically, 0.5 μg of reporter plasmid, 0.2 μg of internal control (CMV-βGal), 10–20 ng of receptor expression vector, 20–100 ng of coactivator expression vector and carrier (Bluescript pKS II) to a total of 1 μg per well were added to the cells. After 12–14 h transfection, the cells were washed twice with PBS and treated with either 10^{-8} M E_2 or carrier (ethanol) for 24 h in phenol-red free DMEM supplemented with antibiotics and 10% stripped FCS. For the treatment with the MEK1 inhibitor PD98059, the cells were washed twice with PBS and treated for 1 h with phenol-red free DMEM supplemented with the antibiotics and 10% stripped FCS containing 50 μM PD98059. After 1 h, the cells were washed and treated with 10^{-8} M E_2 or carrier (ethanol) for 24 h as described previously. Cells were then washed with PBS and harvested in a potassium phosphate lysis buffer containing 1% Triton X-100. Luciferase and β-galactosidase assays were performed as previously described [32]. All the transfection results presented are the average of at least two independent experiments performed in duplicate.

2.3. Antibodies and Western analysis

Proteins were extracted from 293T cells transfected with 10 μg of hERα, mSRC-1, SRA or the control plasmid. Twenty-four hours prior transfection, the cells were split and seeded into 10 cm dishes in DMEM supplemented with antibiotics and 10% complete FCS. Twenty-four hours post-transfection, the cells were harvested in an NP-40-glycerol containing buffer and lysed using three freeze-thaw cycles as previously described [5]. The extracted

proteins were quantified by the Bradford assay, using the Protein assay Dye reagent curve method (Bio-Rad) and the aliquots were quick-frozen and stored at −80 °C. The same amount of quantified proteins for each sample (typically 20–40 μg) were boiled and loaded on a 8% SDS-denaturing polyacrylamide gel. The gel was stained in coomassie blue dye for 15 min and de-stained in 10% acetic acid overnight. For Western blots, the gel was transferred to PVDF transfer membrane (Hybond P, Amersham Pharmacia Biotech) for protein transfer for 2 h at 0.2 A (100 V) in a Tris-glycine containing buffer with 20% methanol. The membrane was blocked overnight in 5% skimmed milk. The membrane was then blotted with specific antibodies for hERα or SRC-1 in TBS-T buffer (Tris-buffered saline Tween) with 5% milk for 1 h. Following three washes in TBS-T, the secondary antibody was added (1:1000) in TBS-T for 1 h and the membrane was washed three times with TBS-T. The immunoblotted proteins were detected using the BM chemiluminesence blotting substrate (POD) (Boehringer Mannheim). The antibody used for mSRC-1 was obtained from Santa Cruz Biotechnology Center (polyclonal mSRC-1 Ab, #C20, sc6096). The antibody used for hERα was obtained from Upstate Biotechnology (monoclonal hERα Ab, #05-394). The secondary antibody used for mSRC-1 (α-goat) was obtained from Santa Cruz Biotechnology Center (anti-goat Ab). The secondary antibody used for hERα (αmouse) was obtained from Roche Diagnostics (anti-mouse Ab).

3. Results

3.1. SRA coactivates ERα and ERβ in a ligand-dependent manner

SRA is a coactivator specific for the steroid receptors. While its coactivation effects have been studied in details on PR [26], less is known about its specific mode of action on other steroid receptors. We investigated the coactivation induced by SRA on the transcriptional activity of the two estrogen receptors, ERα and ERβ. Transient cotransfections of ERα or ERβ along with SRA were performed in COS-1 cells in the absence or in the presence of E_2 in order to study its effect on the transcriptional activity of these receptors on a ERE-TKLuc reporter. Cotransfection of SRA and ERα did not lead to enhancement of transcriptional activity of the receptor in the absence of hormone. Treatment with E_2 induced an eight-fold increase in the transcriptional activity of ERα and addition of SRA further increased by three-fold this ligand-dependent transcriptional activity (Fig. 1A). Similar results were obtained for ERβ (Fig. 1B). The ligand-dependent effect of SRA on the transcriptional activity of the receptors was similar for ERα (3-fold) and ERβ (2.5 fold) (Compare Fig. 1A and B). Similar results were obtained in HeLa and 293T cells (data not shown).

The ligand-dependent coactivation induced by SRA on ER works in a dose-dependent manner (Fig. 1C). Increas-

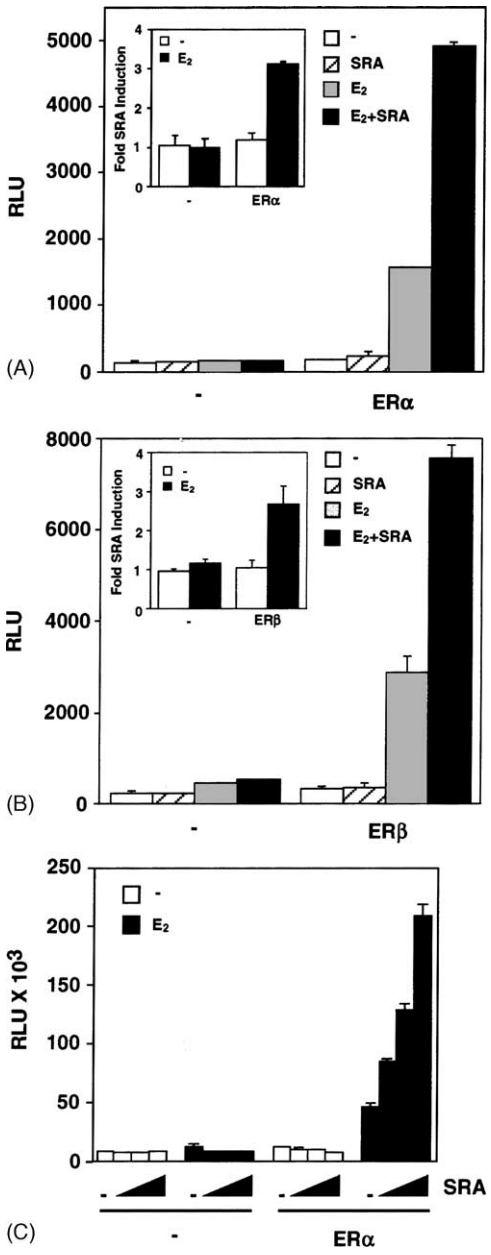

Fig. 1. Coactivation effect of SRA on ERα and ERβ transcriptional activity. (A) Transient transfections demonstrate that addition of SRA in the absence of ligand does not have any effect on the transcriptional activity of ERα. In the presence of ligand, addition of SRA induces a two-fold increase in the ligand-dependent transcriptional activity of ERα. The fold-SRA-induction is represented in the inset. (B) Same experiment performed with ERβ. (C) Transient transfection showing that addition of increasing amounts of SRA induces a proportional increase in the transcriptional activity of ERα (ERα:SRA 1:2, 1:5, 1:10). All the transfections were carried out in COS-1 cells using the 2C-vERE-TKLuc reporter gene and p-CMX-based expression vectors as described in the Materials and Methods section. The results expressed in relative luciferase units (RLU) represent the ratio between the luciferase values and the β-galactosidase control values. Transfected cells were treated with either 10^{-8} M E_2 or carrier (ethanol) for 24 h post-transfection.

ing amounts of cotransfected SRA in COS-1 cells led to an increase in ERα transcriptional activity, suggesting that SRA acts as a limiting factor in mediating ligand-dependent coactivation of ERα.

3.2. Coactivation of ERα and ERβ by coexpression of SRA and SRC

SRA was shown to be part of a large complex containing SRC-1 [26]. Since the coactivation effect of SRC-1 on ERα and ERβ has been well described, we decided to investigate the possible cooperation between the two coactivators in inducing the enhancement of ER transcriptional activity. COS-1 cells were transiently cotransfected with ERα or ERβ along with SRA, SRC-1 or both (Fig. 2A). SRA and SRC-1 each induced a two-fold enhancement of ERα ligand-dependent transcriptional activity (Fig. 2A, left panel). Cotransfection of both SRC-1 and SRA mediated a four-fold induction in the ligand-dependent transcriptional activity of ERα, suggesting that coexpression of both SRC-1 and SRA resulted in a two-fold increase of the SRA effect on ERα-mediated transactivation (Fig. 2A, left panel). The same effect of SRA and SRC-1 was observed on ERβ (Fig. 2A, right panel).

We next investigated whether the coactivation observed when SRA and SRC-1 were coexpressed could result from an SRA-mediated alteration in the levels of SRC-1 or ER expression. To address this question, 293T cells were transiently transfected with SRC-1 or ERα, and SRA or empty vector. The extracted proteins were quantified and subjected to Western analysis using a specific anti-SRC-1 or anti-ERα antibody. No significant change in SRC-1 (Fig. 2B, left panel) or ERα expression (Fig. 2B, right panel) was detected upon addition of SRA, suggesting that overexpression of SRA did not alter the expression level of either SRC-1 or ERα in the context of this assay. COS-1 cells were also used to perform this experiment but the expression level of these proteins in COS-1 cells was too low to be detected by this assay (data not shown).

Transient cotransfections of SRA and ER in COS-1, 293T and HeLa cells revealed that some level of SRA coactivation does not require the addition of exogenous SRC-1 (Fig. 1). To investigate the possibility that other members of the p160/SRC family of coactivators mediate an increase in SRA-induced coactivation of ER, COS-1 cells were transiently cotransfected with ERα, SRA and SRC-2 or SRC-3. Both SRC-2 (Fig. 2C, left panel) and SRC-3 (Fig. 2D, left panel) enhanced the ligand-dependent transcriptional activity of ERα by two-fold. Cotransfection of SRA along with SRC-2 or SRC-3 resulted in a two-fold increase of the SRA effect on ERα (Fig. 2C and D, left panel), as seen with SRC-1. The same two-fold increase in the effect of SRC-2 (Fig. 2C right panel) or SRC-3 (Fig. 2D, right panel) with SRA was observed on the ligand-dependent transcriptional activity of ERβ.

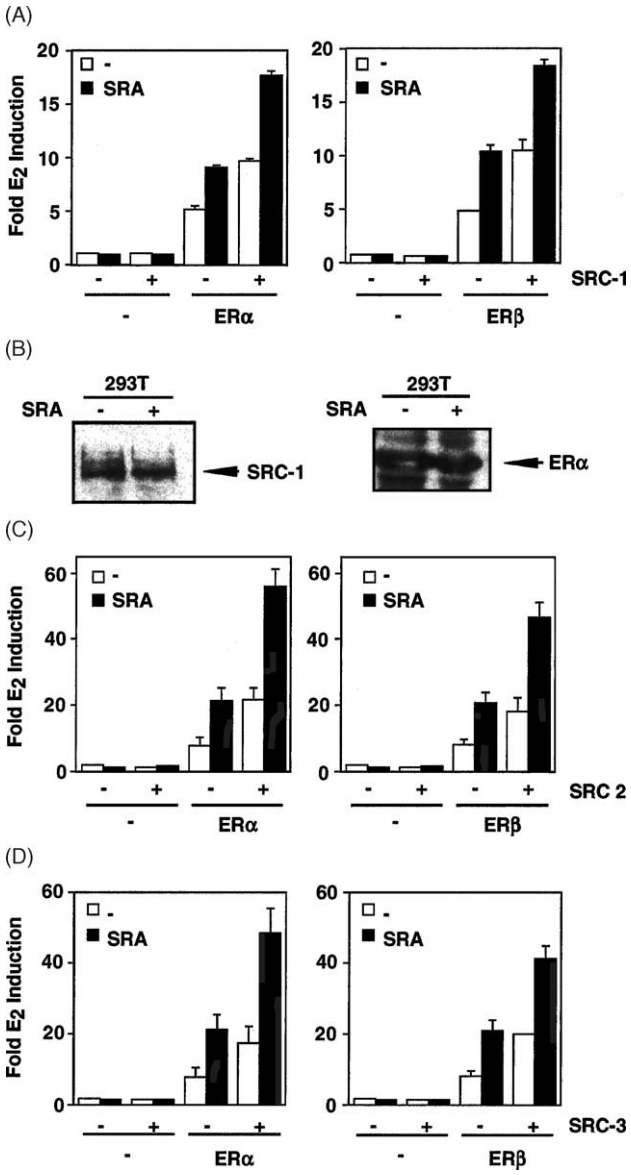

Fig. 2. Coactivation of ERα and ERβ upon SRA and SRC coexpression. (A) Transient transfection that demonstrates that coexpression of SRA and SRC-1 results in a four-fold increase in the ligand-dependent transcriptional activity of both ERα (left panel) and ERβ (right panel). The initial individual coactivation induced by SRA or SRC-1 was of two-fold in presence of ligand for both receptors. The results are expressed in fold E_2 induction (ligand present in all the lanes). (B) Western blot showing that overexpression of SRA in 293T cells does not alter the levels of expression of transfected SRC-1 (left panel) and ERα (right panel). (C) Same experiment as in (A) using SRC-2 instead of SRC-1. (D) Same experiment as in (A) using SRC-3 instead of SRC-1. All the transient transfections were carried out as described in Fig. 1.

3.3. SRA coactivation involves different motifs in ERα and ERβ

SRA was cloned as an AF-1-dependent coactivator for PR [26]. The different regions required for SRA-mediated coactivation for other steroid receptors have not been determined. To investigate the respective involvement of the AF-1

and AF-2 domains of ER in SRA-induced coactivation, we tested the effect of SRA on the transcriptional activity of several ER mutants. The ERα mutants used included CDEFα, which lacks the "AB" portion (AF-1) of ERα, ABCα, which lacks the LBD (AF-2) of ERα and ERα^{L539A}, a mutant in which leucine 539 was changed to an alanine. The L539A mutation completely abolishes the AF-2 activity of the receptor by preventing helix 12 from interacting with coactivators [19]. COS-1 cells were transiently cotransfected with wild-type ERα or ERα mutants, along with SRA. CDEFα still responded to E_2 stimulation and addition of SRA in presence of E_2 further increased the transcriptional activity of the mutant by two-fold, similarly to the wild-type ERα (Fig. 3A). As shown for wild-type ERα, SRA had no effect on the basal transcriptional activity of CDEFα in the absence of ligand. The behavior of CDEFα mutant in the presence of SRA was identical to the one observed for the wild-type ERα, indicating that SRA might have the ability to coactivate the receptor through the AF-2 domain alone in the presence of E_2, without requiring the AF-1 domain of the receptor.

In order to investigate whether E_2 was required to mediate SRA effect, we used ABCα, a deletion mutant that can no longer bind the ligand due to the absence of the LBD, but that can still dimerize when bound to DNA. In the absence of ligand, SRA does not have any effect on the basal transcriptional activity of ABCα mutant (Fig. 3A). As expected, treatment with E_2 did not induce transcriptional activation of the mutant receptor. Surprisingly, cotransfection of SRA in the presence of E_2 increased the transcriptional activity of the ABCα mutant by two-fold, despite the fact that this mutant did not have the ability to bind E_2 (Fig. 3A). Cotransfection of SRA in the presence of ligand also increased the transcriptional activity of ERα^{L539A} mutant by two-fold, suggesting that SRA might also possess the ability to coactivate ERα through its AF-1 domain without requirement for the AF-2 domain (Fig. 3A). However, this AF-1 mediated effect of SRA on ABCα and ERα^{L539A} seems to require the presence of E_2, despite the fact that these mutants did not show any ligand-induced activity. For all the mutants tested, SRA induced a two-fold increase in transcriptional activity of the receptor in the presence of E_2, an effect that was similar to what was observed for the wild-type ERα (Fig. 3A, inset).

To investigate the domains of ERβ involved in SRA-induced transcriptional activity of the receptor, the same deletions and mutations were generated in ERβ: CDEFβ, ABCβ and ERβ^{L445A}, an AF-2 defective mutant. These receptors were transiently cotransfected in COS-1 cells along with SRA (Fig. 3B). The E_2-induced transcriptional activation of the CDEFβ mutant was further increased by two-fold upon addition of SRA (Fig. 3B). This ligand-dependent SRA effect was identical to the one observed for the wild-type ERβ suggesting that, like ERα, SRA might have the ability to increase the E_2-dependent activity of the receptor via the AF-2 domain without requirement for the AF-1 domain. ABCβ and ERβ^{L445A} mutants did not show any E_2-induced

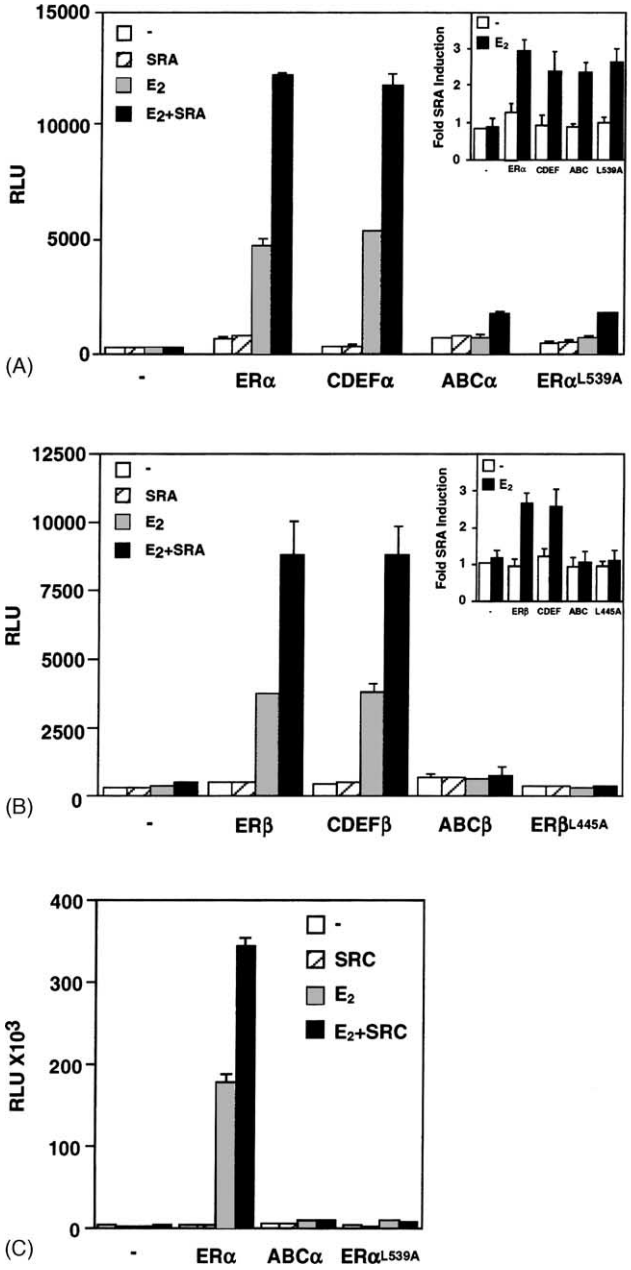

Fig. 3. Involvement of different domains of ER in SRA coactivation of ER transcriptional activity. (A) Transient transfection demonstrates that specific domains of ERα play specific roles in SRA coactivation. SRA induced a two-fold coactivation of all the mutants tested, in the presence of ligand (inset, expressed in fold-SRA-induction). (B) Same experiment with the deletion and point mutants in ERβ. SRA induced a two-fold coactivation of the wild-type and the CDEFβ mutant only, in the presence of ligand. The ABCβ and ERβ^{L445A} mutants were not activated by SRA even in the presence of ligand (inset, expressed in fold-SRA-induction). (C) Transient transfection is showing the effect of SRC-1 on the different mutants in ERα. SRC-1 induced a two-fold increase in the transcriptional activity of ERα only, in the presence of ligand. All the transient transfections were carried out as described in Fig. 1.

transcriptional activity due to the absence of the LBD and functional AF-2, respectively (Fig. 3B). SRA did not have any effect on the activity of these mutants, either in the absence or presence of E_2. Thus, SRA induced a two-fold increase in transcriptional activity of the wild-type ERβ and CDEFβ in presence of E_2. No significant increase in transcriptional activity due to SRA was observed for ABCβ and ERβ^{L445A}, either in the presence or absence of E_2 (Fig. 3B, inset).

To determine whether the SRA-mediated coactivation observed on ABCα and ERα^{L539A} in the presence of ligand resulted solely from overexpression of SRA, or if this effect could also be observed upon overexpression of SRC-1, COS-1 cells were transiently cotransfected with SRC-1 and ERα or the mutant receptors. Overexpression of SRC-1 did not induce any ligand-dependent coactivation of ABCα or ERα^{L539A} (Fig. 3C), suggesting that the SRA effect observed for these mutants in the presence of E_2 is specific for the RNA coactivator.

3.4. SRA requires the presence of S^{118} in the AF-1 of ERα to mediate full activation

The presence of an intact S^{118} within the AF-1 of ERα was shown to be essential for full activity of the receptor both in the absence and presence of E_2, an effect that is mediated through its phosphorylation [22]. Since SRA seems to partially mediate its action through the AF-1 of ERα, we investigated whether S^{118} was required for this effect. COS-1 cells were transiently cotransfected with SRA, along with ERα or a mutant in which S^{118} was replaced with an alanine residue (ERα^{S118A}) (Fig. 4). This mutant harbors a decreased phosphorylation state of ERα that diminishes the transcriptional activity of the receptor, even in the presence of E_2 [24]. The transcriptional activity of ERα^{S118A} in the presence of E_2 was significantly lower (2.3-fold) compared to the activity observed for the wild-type ERα (7.3-fold) (Fig. 4). Mutation of S^{118} to alanine caused a significant decrease in E_2-dependent SRA effect (1.3-fold induction) compared to ERα wild-type (2.5-fold) (Fig. 4, inset).

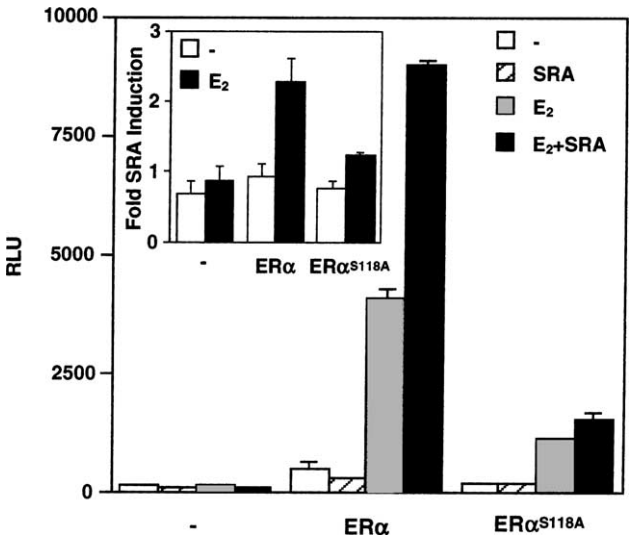

Fig. 4. Requirement of serine residue 118 in ERα AF-1 for coactivation by SRA. Transient transfection showing that mutation of serine residue 118 (S^{118}) to alanine residue in ERα AF-1 results in a significant decrease in the ligand-dependent SRA-induced coactivation of ERα transcriptional activity. All the transient transfections were carried out as described in Fig. 1.

3.5. MAPK activation leads to SRA effect on ERα AF-1 in absence of ligand

The presence of an intact S^{118} in ERα AF-1 is required for full SRA-induced coactivation of ERα. S^{118} is phosphorylated by MAPK, whose activity is induced by various extra cellular stimuli, including non-genomic action of E_2 [33]. The effect of SRA on ABCα and ERα^{L539A} mutants was unexpected since SRA could mediate AF-1-induced coactivation of the mutant receptors only in the presence of E_2 despite the fact that these mutants do not show estrogenic responses. We were thus interested in testing whether this SRA effect in the presence of E_2 could be mediated by MAPK activation and the potential phosphorylation of S^{118} in ERα AF-1. To this effect, COS-1 cells were transiently cotransfected with ERα, ABCα or ERα^{S118A} along with SRC-1, H–Ras^{V12} (a dominant active Ras that induces MAPK pathway) or both. All these experiments were carried out in steroid-deprived serum and in the absence of E_2 stimulation to make sure that the effects observed were caused solely by MAPK-induced phosphorylation and ligand-independent coactivator interactions (Fig. 5A). Transient cotransfection of ERα along with H–Ras^{V12} led to a two-fold increase in ligand-independent activity of the receptor (Fig. 5A). Addition of SRA to ERα and H–Ras^{V12} further increased the activity of the receptor by two-fold (Fig. 5A). Addition of SRC-1 to H–Ras^{V12} and SRA contributed to further increase this ligand-independent activation of ERα by seven-fold as compared to what was initially observed in the absence of MAPK stimulation (Fig. 5A). The same effects of H–Ras^{V12}, SRA and SRC-1 were observed on the ABCα mutant, suggesting that the increase in transcriptional activity observed for wild-type ERα is mediated through its AB domain. Upon transfection of ERα^{S118A}, no increase in the activity of the receptor was observed in the presence of H–Ras^{V12}. Addition of SRA or SRC-1 did not have any effect on the transcriptional activity of ERα^{S118A}, suggesting that the ligand-independent signaling induced by SRA in the presence of MAPK activation could be mediated through the presence of S^{118} in ERα AF-1.

To further verify that the effect of SRA observed on ABCα and ERα^{L539A} in the presence of ligand was caused by MAPK activation, we used a MAPK inhibitor, PD98059, and asked whether it abolishes this ligand-dependent effect of SRA on the two mutants. COS-1 cells were transiently cotransfected with ERα or ABCα along with SRA and stimulated with E_2 in the absence or in the presence of

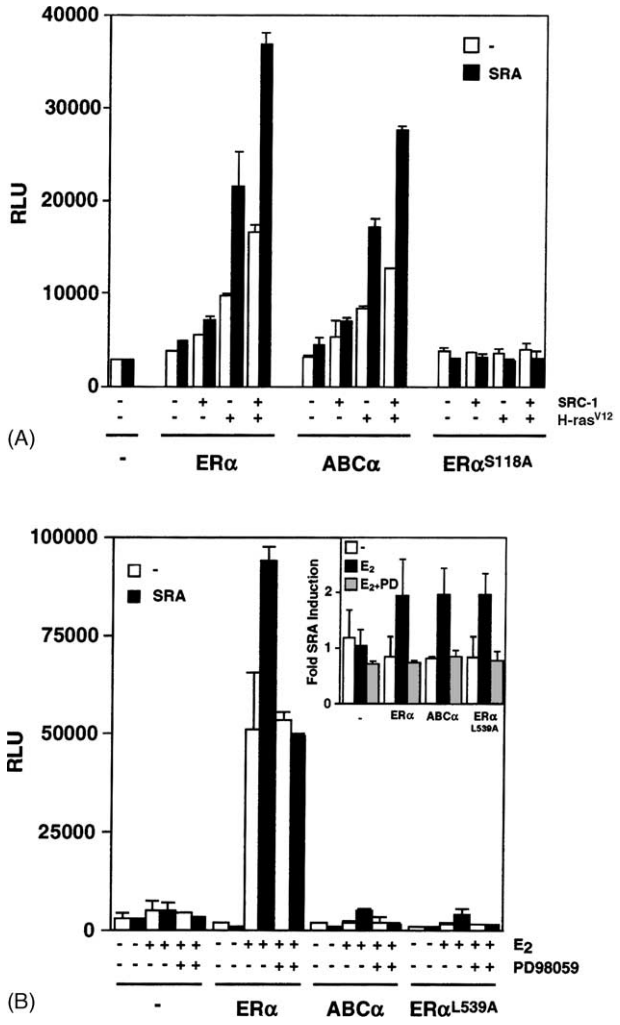

Fig. 5. Activation of MAPK induces the ligand-independent coactivation of ERα AF-1 by SRA. (A) Transient transfection performed in the absence of ligand showing that SRA induces the coactivation of ERα and ABCα mutant in presence of MAPK activation through cotransfection of H-ras^{V12}. Mutation of S^{118} in ERα AF-1 abolishes the effect of H-ras^{V12}, SRA and SRC-1 in the absence of ligand. (B) Transient transfections showing that treatment with a MEK1 inhibitor (PD98059) abolishes the ligand-dependent SRA effect. All the transient transfections were carried out as described in Fig. 1. with the exception that treatment with PD98059 (50 μM) was performed for 1 h prior to E_2 or carrier treatment.

PD98059. Treatment with the MAPK inhibitor completely abolished the ligand-dependent effect of SRA on ERα and the ABCα mutant (Fig. 5B).

4. Discussion

In this work, we describe the coactivation effect of SRA on the transcriptional activity of ERα and ERβ. We showed that SRA coactivates ERα and ERβ in a ligand-dependent manner. We also demonstrated that the transcriptional activity of ERα can be enhanced by SRA in a ligand-independent manner through the AF-1 domain of the receptor. The data indicates that SRA mediates the coactivation of ERα via multiple molecular mechanisms, either ligand-dependently or independently through the AF-2 or the AF-1, respectively. The fact that this AF-1-dependent effect of SRA was not observed on ERβ denotes the ability of SRA to mediate differential activation of ERα and ERβ. The finding that SRA-mediated AF-1-dependent coactivation of ERα led us to investigate the possible involvement of S^{118} in ERα AF-1 in the mechanism of SRA action. We found that an intact S^{118} was required for the complete coactivation of ERα by SRA. We also showed that activation of MAPK induced ligand-independent coactivation of ERα by SRA, a mechanism that is independent of the AF-2 activity of the receptor. Finally, we showed that SRA is unable to rescue the loss of activity of the S^{118} ERα mutant in response to H–Ras^{V12}, suggesting that phosphorylation of S^{118} by MAPK participates in the ligand-independent effect of SRA on ERα.

In this study, we have investigated the coactivation effect of SRA on ERα and ERβ transcriptional activity. While SRA mediated a two- to three-fold induction of ERα and ERβ transcriptional activity in presence of E_2, no significant alteration in ER activity was observed upon overexpression of SRA in the absence of ligand. This finding is similar to what was previously reported for PR, yet it differs from the effect observed on GR, where SRA was shown to mediate coactivation of full-length GR even in the absence of the ligand [26].

The presence of an intact AF-2 is sufficient for the induction of the ligand-dependent coactivation of both ERα and ERβ by SRA despite the fact that SRA was originally cloned as an AF-1-dependent coactivator. As previously suggested, SRA might be recruited to ERs via other coactivators that interact in a ligand-dependent fashion with the AF-2 of the receptor, such as SRC-1. However, the specific effect of SRA on several mutants of ERα and ERβ revealed a differential role for SRA in mediating ERα and ERβ coactivation.

While the SRA-induced coactivation of ERβ appears to be strictly ligand- and AF-2-dependent, a significant and consistent level of AF-2-independent coactivation of ERα was detected, suggesting that SRA might as well mediate AF-1-dependent activity on ERα. The differential effects of SRA on ERα and ERβ AF-1 were not surprising considering the fact that both receptors share less than 15% homology in their AB region. In agreement with the lack of homology observed in this domain, it had already been established that ERα and ERβ show significant differences in their AF-1 activities. While SRC-1 had been shown to associate with ERβ AF-1 upon phosphorylation of serine residues 106 and 124 in ERβ AF-1 [19], this effect does not involve SRA coactivation (data not shown). The SRA coactivation of ERα AF-1 suggests that SRA might be part of a coactivator complex that is recruited to the AF-1 of the receptor in a ligand-independent manner. This complex of coactivators may not be the same as the one previously isolated and shown to also contain SRC-1 or SRC-2 [26,28]. The fact that overexpression of SRC-1 did not have any effect on the

transcriptional activity of ERα mutants both in the presence and in the absence of the ligand suggests that SRC-1 might in fact not be involved in the SRA-containing complex recruited to the AF-1. The indirect recruitment of SRA to the AF-1 led us to postulate that SRA might represent the bridging factor that allows synergy between the AF-1 and the AF-2. Since SRC-1 is recruited to ERα in a ligand-dependent manner via the AF-2, it can further be thought that SRA might allow the cross-talk between the AF-1 and the AF-2 of the receptor leading to the synergism of their individual activities. However, since overexpression of both coactivators does not allow us to discern between an additive or a synergistic effect, we cannot speculate on the possible synergism potentially induced by SRA on ERα AF-1 and AF-2.

The finding that SRA coactivation of ERα AF-1 was generated only upon E_2 treatment was unexpected considering the fact that the two mutants used to investigate this effect (ABCα and ERα^{L539A}) did not possess the ability to generate a ligand response. The most likely interpretation is that E_2 itself induces rapid and sustained activation of MAPK, which in turn phosphorylates serine residues in ERα AF-1. This could suggest that phosphorylation of ERα AF-1 induces a favorable interface for a SRA-containing complex in the absence of a functional AF-2.

S^{118} in ERα AF-1 is a target for MAPK-induced phosphorylation in the absence of ligand. The integrity of S^{118} was required for SRA-mediated coactivation of the full-length ERα upon MAPK activation in the absence of ligand. One possible explanation for this effect suggests that even in the context of the full-length receptor, SRA might in part mediate its coactivation function via the presence of phosphorylated S^{118} in ERα AF-1. While phosphorylation of S^{118} has been shown to be essential for the full activity of the receptor both in the presence and in the absence of ligand, the functional significance of this phosphorylation event had not been clearly established. One suggestion was that phosphorylation of S^{118} might help recruit the p68 RNA helicase, a weak coactivator of ERα AF-1 [25]. An alternative hypothesis could be that the indirect recruitment of SRA to the AF-1 of ERα might represent the connection between S^{118} phosphorylation and the full activity of the receptor by helping recruit other coactivator complexes to the AF-1 of ERα.

Taken together, our data support an important role for SRA in the coactivation of ER. The finding that SRA induces differential coactivation of ERα and ERβ AF-1 incites us to postulate that it might serve a specific role in a particular receptor context, which could lead to tissue and cell specific function of SRA. In this regard, it will also be of interest to investigate how SRA works in the context of ER heterodimers.

Finally, the finding that SRA coactivates ERα upon MAPK activation in the absence of ligand binding is of important therapeutic relevance. Aberrant activation of MAPK has been reported in human breast tumors [34], leading to an upregulation of ER AF-1 activity in ER^+ breast tumors. It can thus be hypothesized that SRA might represent one of the key components in mediating this aberrant upregulation of ER activity. This is further supported by the finding that SRA expression was found to vary during breast tumor progression [30,31]. Additional studies will be required to elucidate the complete molecular mechanism by which SRA mediates the coactivation of ER both in a ligand-dependent and independent manner. A better comprehension of its molecular mode of action will ultimately help us develop a better scheme of ER coactivation and this might help understand some endocrine diseases associated with ER.

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