

Ligand-Independent Recruitment of SRC-1 to Estrogen Receptor β through Phosphorylation of Activation Function AF-1

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Summary

The estrogen receptors (ERs) α and β possess a constitutive N-terminal activation function (AF-1) whose activity can be modulated by kinase signaling pathways. We demonstrate here that phosphorylation of AF-1 by MAP kinase (MAPK) leads to the recruitment of steroid receptor coactivator-1 (SRC-1) by ER β in vitro. Enhancement of the interaction between SRC-1 and ER β AF-1 is also observed in vivo in cells either treated with EGF or expressing activated Ras. Two serine residues in ER β AF-1, of which one is contained within a motif present in other steroid receptors, are critical for physical interaction with SRC-1 and transcriptional activation. Our results establish a role for nuclear receptor phosphorylation in the recruitment of SRC-1 and provide a molecular basis for ligand-independent activation by ER β via the MAPK pathway.

Introduction

The estrogenic signal is now known to be transduced by two estrogen receptors (ER α and β) that are members of the superfamily of nuclear receptors (Green et al., 1986; Greene et al., 1986; Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The two ERs share many functional characteristics based on their well-conserved modular structure. In particular, ER α and β contain two activation functions (AF) residing in the NH₂-terminal A/B domain (AF-1) and the COOH-terminal end of the E domain (AF-2), respectively. AF-2 is responsible for hormone-dependent activation through recruitment of coactivator proteins including members of the steroid receptor coactivator (SRC) family (reviewed in Glass et al., 1997). On the other hand, AF-1 activity is constitutive and ligand independent (Kumar et al., 1987; Berry et al., 1990; Metzger et al., 1995). While SRC family members were recently shown to interact weakly with the N-terminal region of ER α and the progesterone receptor (PR) in vitro (Lavinsky et al., 1998; Oñate et al., 1998; Webb et al., 1998), the precise molecular mechanism through

which AF-1 participates in transcriptional activation remains to be determined.

In addition to the classical hormone activation pathway, a number of steroid receptors including ER α and β have been shown to be activated by nonsteroidal agents including dopamine, growth factors, and PKA activators (reviewed in Weigel and Zhang, 1998). This ligand-independent activation is presumed to be closely related to phosphorylation of steroid receptors by cellular protein kinases. Stimulation of cellular activity by growth factors or cAMP analogs results in ER-dependent transcriptional activation accompanied by an increased receptor phosphorylation (Aronica and Katzenellenbogen, 1993; Bunone et al., 1996). In particular, Ser-118 in the A/B region of human ER α was shown to mediate epidermal growth factor (EGF) activation through the Ras-MAP kinase (MAPK) signaling cascade (Kato et al., 1995; Bunone et al., 1996). Similarly, ER β can be activated by the Ras pathway, and this effect is mediated through Ser-124 in the A/B region of mouse ER β (Tremblay et al., 1997). The presence of several additional Pro-directed kinase sites within the A/B region of both ERs suggests that differential phosphorylation of the AF-1 domain may modulate the activity of ERs in response to diverse physiological cues. Here we examine the mechanisms by which phosphorylation of the ER β AF-1 domain via the MAPK cascade leads to transcriptional activation. We show that phosphorylation of MAPK sites within the AF-1 stimulates a ligand- and AF-2-independent interaction between ER β and SRC-1. Our results demonstrate that phosphorylation of ER β AF-1 regulates coactivator recruitment and gene activation by nonsteroidal activators.

Results

SRC-1 Induces AF-2-Independent ER Transcriptional Activation

Our previous observations that SRC-1 could stimulate ER β activity in the absence of ligand prompted us to further investigate the mechanisms underlying this effect (Tremblay et al., 1997). In the absence of E₂, SRC-1 increased in a dose-dependent manner the transcriptional activity of ER β in transfected Cos-1 cells. This effect was not cell- or reporter-specific since a similar increase in ER β activity was detected on various reporters such as pS2Luc and ERE₃TKLuc and in other cell lines including HeLa and 293 T (data not shown). Based on the previous observation that SRC-1 could interact with steroid receptors in a ligand- and AF-2-independent manner (Takeshita et al., 1996; Henttu et al., 1997; Jeyakumar et al., 1997), we tested whether AF-2 was necessary to mediate the SRC-1 effect on basal ER β activation using the differential ability of the partial antagonist 4-hydroxytamoxifen (OHT) and the pure antiestrogen EM-652 to block only AF-2 or both AF-1 and AF-2 functions. As shown in Figure 1, EM-652 strongly impaired the SRC-1-mediated basal ER β activity in Cos-1 cells while OHT treatment had no effect.

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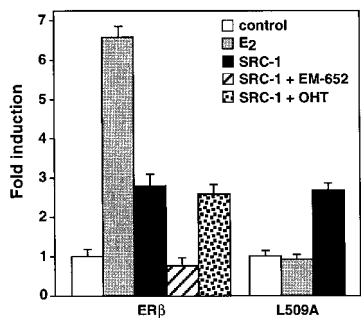


Figure 1. Basal ERβ Transcriptional Activation by SRC-1 Is AF-2 Independent

Pure antiestrogen EM-652 but not the partial antagonist OHT inhibits basal ERβ transcriptional activation by SRC-1. Cos-1 cells were transfected with ERETKLuc along with equivalent amounts of ERβ and SRC-1 expression plasmids and incubated with 100 nM OHT or EM-652. Basal activity of an ERβ AF-2 mutant is induced by SRC-1. Cos-1 cells were transfected with ERETKLuc reporter and equivalent amounts of ERβ or ERβ L509A AF-2 mutant and SRC-1 expression plasmids. Cells were then treated with 10 nM E₂ or left untreated for 16 hr prior to harvest.

The same pattern of inhibition was obtained in HeLa cells with the pS2Luc reporter (data not shown). To test this possibility further, we used an ERβ AF-2-deficient mutant (ERβ^{L509A}) that is transcriptionally inactive in the presence of E₂ (Figure 1). SRC-1 could still activate ERβ^{L509A} in the absence of ligand. Taken together, these data demonstrate that the transcriptional effect of SRC-1 on unliganded ERβ occurred in an AF-2-independent fashion.

The Ligand-Independent Activation by SRC-1 Resides in the AF-1-Containing Region of ERβ and Is Transposable

In an attempt to delineate the region responsible for the SRC-1 activation of unliganded ERβ, truncated and chimeric forms of ERβ were used in transfection experiments (Figure 2A). Transcriptional activities of both the long (ERβ; aa 1–549; GenBank accession #AF067422) and short (ERβ-S; aa 65–549; GenBank accession #U81451) forms of unliganded ERβ were increased to a similar extent by SRC-1, thus excluding region 1–64 present in the long form of ERβ from being involved in the SRC-1 activation (Figure 2B). When the LBD of ERβ-S was deleted, the transcriptional activity of the resulting ABCβ construct was still induced by cotransfected SRC-1. In contrast, activity of an ERβ mutant missing the N-terminal region (CDEFβ) was not stimulated by SRC-1 in the absence of E₂. These results illustrate that the AF-1-containing A/B domain of ERβ (aa 65–167) was necessary and sufficient to mediate SRC-1 activation in the absence of E₂. The basal activity of wild-type and truncated variants of ERα remained unchanged under the same conditions. Interestingly, when the A/B region of ERα was replaced by A/β, the resulting ERβα chimera became as responsive to SRC-1 as wild-type ERβ in the absence of E₂ (Figure 2B). Conversely, the replacement of A/β by A/βα (ERαβ chimera) resulted in a loss of activation by SRC-1. The effect of SRC-1 activation on the ERβ AF-1 is thus transposable. ERαβ

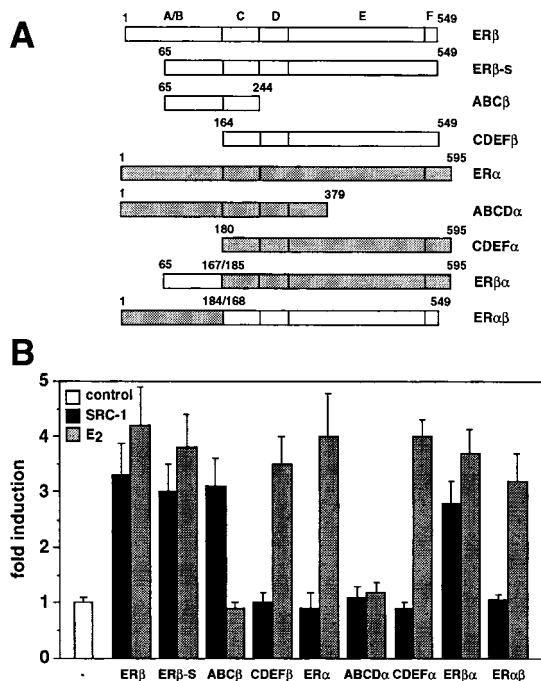


Figure 2. The Ligand-Independent Effect of SRC-1 Resides in the A/B Region of ERβ and Is Transposable

(A) Representation of the various constructs used. Numbering of mouse ERβ is relative to its longest form.

(B) Cos-1 cells were transfected with expression plasmids encoding the various ERα and β derivatives and SRC-1, and transcriptional activities were measured on an ERETKLuc reporter. Cells were treated with 10 nM E₂ or left untreated for 16 hr prior to harvest.

and ERβα chimera were both responsive to E₂, demonstrating that domain swapping had no effect on receptor integrity. Similar results were obtained with constructs in which A/β or A/βα were linked to the DBD of the yeast activator Gal4. Both Gal4-ER chimeras showed intrinsic transcriptional activity conferred by AF-1, but only the Gal4-A/β fusion activity was enhanced by SRC-1, whereas Gal4 DBD alone or Gal4-A/βα was not (data not shown).

Interaction of SRC-1 with ERβ AF-1

To determine the precise boundaries of the AF-1 determinants essential for ligand-independent activation by SRC-1, we generated a series of constructs that contained progressive N- or C-terminal deletions within the ERβ A/B domain (Figure 3A). The various ERβ mutants were tested in transient transfection experiments where the region between aa 104 and 122 was shown to be critical for SRC-1 activation of the unliganded receptor. This observation was confirmed by the internal deletion mutant ERβΔ104–122 that failed to respond to SRC-1 (Figure 3A). We next tested whether this region could mediate interaction with SRC-1. As demonstrated by GST pull-down assay with bacterially purified GST-A/β fusion and in vitro ³⁵S-labeled SRC-1 protein, the A/β region (aa 65–167) of ERβ was able to interact with SRC-1 (Figure 3B). However, when amino acids 104–122 were removed from GST-A/β, the resulting GST-

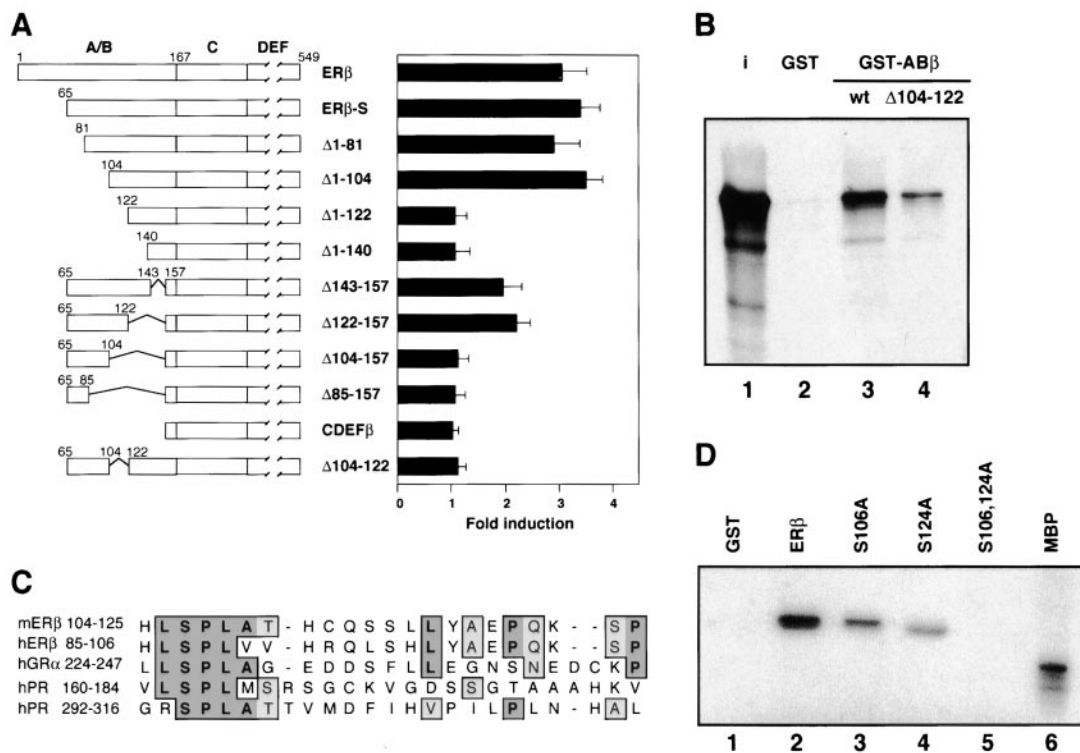


Figure 3. Interaction of SRC-1 with ERβ AF-1

(A) Region 104–122 of ERβ AF-1 mediates ligand-independent activation by SRC-1. Various N- and C-terminal deleted forms of ERβ within the A/B region were tested in transfection on an ERETCLuc in Cos1 cells in the presence of SRC-1 expression vector. (B) SRC-1 associates with the A/B region of ERβ in vitro. Pull-down experiments were performed with in vitro translated and ³⁵S-labeled SRC-1. The input lane (i) represents 5% of the labeled SRC-1 used in each experiment. (C) Identification of two consensus Pro-directed kinase sites at positions 106 and 124 in ERβ AF-1 of which the motif comprising Ser-106 is conserved with other steroid receptors within the A/B region. Numbering of human ERβ is relative to its longest form, and of PR to PR-B. (D) Ser-106 and Ser-124 are target sites for MAPK. GST fusions of wild-type and serine-mutated ERβ (aa 96–130) were in vitro phosphorylated by Erk2. MBP was added as a MAPK control substrate.

A/BΔ104–122 showed a severely diminished association with SRC-1.

Examination of the ERβ sequence within aa 104–125 revealed the presence of two consensus Pro-directed kinase sites at positions 106 and 124 (Figure 3C). Ser-124 was previously shown to mediate Ras activation of ERβ (Tremblay et al., 1997) while Ser-106 is part of a motif shared with other steroid receptors within the A/B region (Figure 3C). Interestingly, the corresponding Ser residues in the glucocorticoid receptor (GR) and PR have already been identified as putative Pro-directed kinase sites (Krstic et al., 1997; Zhang et al., 1997). Given the potential role played by phosphorylation in steroid receptor activation, we examined the ability of MAPK to phosphorylate this region of the receptor in vitro. As shown in Figure 3D, the GST fusion of an ERβ peptide (aa 96–130) was readily phosphorylated by MAPK, whereas mutation of either Ser-106 or Ser-124 to Ala reduced the extent of phosphorylation by half as compared to wild type. The double-mutant ERβ^{S106,124A} peptide was not phosphorylated by MAPK.

MAPK-Mediated Phosphorylation of ERβ AF-1 Enhances the Recruitment of SRC-1

To investigate whether phosphorylation of Ser-106 and Ser-124 could regulate SRC-1 interaction with ERβ AF-1,

we first used in vitro MAPK-phosphorylated GST-ABCβ fusion proteins in pull-down experiments. As shown in Figure 4A, phosphorylation of GST-ABCβ led to a significant increase in the interaction with ³⁵S-labeled SRC-1 (compare lanes 3 and 4), demonstrating that phosphorylation of AF-1 directly influenced SRC-1 recruitment by ERβ. Mutation of Ser-106 (ERβ^{S106A} and ERβ^{S106,124A}) abolished the increased interaction between SRC-1 and ERβ AF-1 observed upon phosphorylation. However, mutant ERβ^{S124A} showed only a partial impairment in SRC-1 recruitment as compared to wild type. We next assessed the effect of phosphorylation of the ERβ AF-1 in regulating interaction with SRC-1 in vivo. Treatment of transfected cells with PD98059, a selective inhibitor of MAPK activation, completely abrogated the SRC-1-mediated activation of unliganded ERβ, while the use of staurosporin, which inhibits protein kinase C, had no significant effect (Figure 4B). The contribution of both Ser-106 and Ser-124 Pro-directed kinase sites toward a full response of unliganded ERβ to SRC-1 was also tested. Mutation of both Ser residues (ERβ^{S106,124A}) resulted in a complete loss of ERβ activation by SRC-1 while mutation of either Ser-106 or Ser-124 alone showed only a partial response (Figure 4B), mimicking the results obtained in the in vitro interaction study. In contrast, mutation of Ser-158, which is located outside the

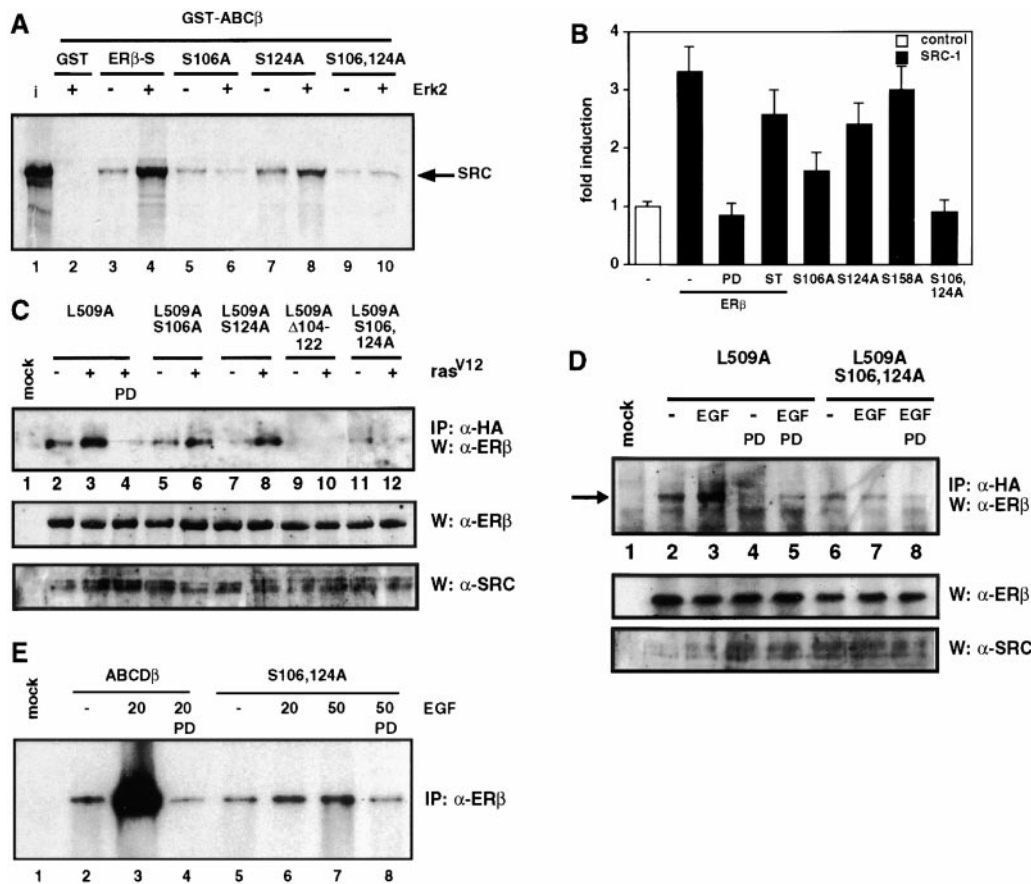


Figure 4. MAPK-Mediated Phosphorylation of ERβ AF-1 Increases SRC-1 Recruitment In Vitro and In Vivo

(A) Phosphorylation of ERβ AF-1 increases the binding to SRC-1 in vitro. Wild-type and mutated GST-ABCβ fusions bound to glutathione beads were incubated in the presence (+) or absence (-) of Erk2 and subjected to pull-down assay with in vitro translated and ³⁵S-labeled SRC-1. Equal amounts of proteins were loaded except for the input (i), which represents 5% of proteins used.

(B) Effects of kinase inhibitors and point mutations on the transcriptional activity of ERβ by SRC-1. Cos-1 cells were transfected with ERETKLuc reporter and ERβ (aa 65–549) or ERβ AF-1 mutants in the absence or presence of SRC-1 expression vector. In the case of kinase inhibitors, cells were treated either with 50 μM PD98059 (PD) or 1 μM staurosporin (ST) 20 min before harvest.

(C) ERβ AF-1 recruits SRC-1 in vivo. 293 T cells were cotransfected with expression vectors encoding HA-tagged SRC-1 and AF-2-defective (L509A) ERβ mutants in the absence or presence of constitutively activated Ras (rasV12) expression vectors. Cells were fed with growth medium for 48 hr and harvested 30 min following treatment with 50 μM PD or vehicle alone. The immunoprecipitates were subjected to Western analysis using an anti-ERβ antibody. As a control, whole-cell extracts of each sample were analyzed by Western blotting to monitor the amounts of ERβ (α-ERβ) and SRC-1 (α-SRC-1) expressed in transfected cells.

(D) EGF induces recruitment of SRC-1 by ERβ AF-1. 293 T cells were transfected with HA-tagged SRC-1 and AF-2-defective (L509A) ERβ mutant expression vectors. Thirty minutes prior to harvest, cells were treated with 20 ng/ml EGF, 50 μM PD, or left untreated. Cell lysates were then analyzed by immunoprecipitation and Western blotting as in Figure 4C.

(E) In vivo phosphorylation of ERβ AF-1. Subconfluent 293 T cells were transfected with wild-type or mutated ABCDβ (aa 65–274) expression plasmids, and refed with DMEM. Forty-eight hours later, the transfected cells were metabolically labeled with [³²P] orthophosphate and treated with EGF (20 and 50 ng/ml) or 50 μM PD, or left untreated. Cells were harvested and subjected to immunoprecipitation with an antibody to ERβ.

mapped SRC-1 interaction domain, has no effect on the enhancement of ERβ activity by SRC-1. The interaction between ERβ and SRC-1 in the absence of hormone was also demonstrated in vivo and shown to be influenced by factors previously shown to change the phosphorylation status of steroid receptors. As shown in Figure 4C (lanes 2 and 3), coimmunoprecipitation of ERβ AF-2-deficient mutant with SRC-1 was significantly increased in cells expressing activated Ras. This process is dependent on MAPK activity since PD98059 completely abrogated the SRC-1/ERβ interaction to a point below the unstimulated state (compare lanes 4 and 2). While mutations of

Ser-106 or Ser-124 had only a partial effect, deletion of residues 104–122 or mutation of both Ser residues (S106,124A) totally disrupted the Ras-mediated increase in interaction between ERβ and SRC-1 (Figure 4C). This observation suggests that Ser-106 and Ser-124 are both required in vivo to fully recruit SRC-1. In addition, when cells were treated with EGF (Figure 4D) or IGF-1 (data not shown), the in vivo interaction between SRC-1 and ERβ was also enhanced, thus mimicking the results obtained in the presence of activated Ras. Again, mutation of Ser-106 and Ser-124 or treatment of cells with the PD98059 completely abrogated the stimulatory effect

of EGF on SRC-1/ER β interaction (Figure 4D). The observation that PD98059 abolished the basal interaction of SRC-1 with the receptor suggests that a fraction of ER β is undergoing phosphorylation in cells under basal conditions. Immunoprecipitation of wild-type and Ser-mutated ABCD β truncated receptors from *in vivo* orthophosphate-labeled cells revealed that ER β is indeed phosphorylated under basal conditions and that treatment of the transfected cells with EGF significantly stimulates phosphorylation of the truncated receptor (Figure 4E). The observation that PD98059 considerably reduced the extent of ER β phosphorylation (compare lanes 2 and 4) supports a role for MAPK in the *in vivo* phosphorylation of the receptor. The importance of both Ser-106 and Ser-124 in ER β phosphorylation was evidenced by a severe diminution in the EGF-induced hyperphosphorylation state of ER β ^{S106,124A} mutant as compared to wild type.

Discussion

In this report, we explored the molecular mechanisms underlying ligand-independent activation of a nuclear receptor, ER β , by the coactivator SRC-1. Our work led to the identification of a novel SRC-1 interaction domain within ER β AF-1, whose activity can be upregulated by phosphorylation. Thus, this study demonstrates that phosphorylation of the AF-1 domain of a member of the nuclear receptor superfamily enhances the recruitment of a steroid receptor coactivator (SRC-1) and provides a molecular basis for ligand-independent activation of ER β via the MAPK cascade.

SRC-1 has been described as a coactivator that interacts and enhances the transcriptional activity of a number of nuclear receptors in a ligand- and AF2-dependent manner (Oñate et al., 1995). Based on three different approaches, enhancement of ER β activation by SRC-1 in the absence of ligand was found to be independent of AF-2. First, the partial antiestrogen OHT had no appreciable effect on SRC-1-induced unliganded ER β activity while the pure antiestrogen EM-652 completely abolished this effect. This observation strengthens the need for pure antiestrogens in breast cancer therapy where all aspects of ER-regulated gene expression, including coactivator-mediated hormone-dependent as well as hormone-independent activation pathways, must be regarded as direct targets for antiestrogen action. Second, transcriptional activity of ER β ^{L509A}, an AF-2-defective mutant, was comparable to wild-type ER β in the presence of SRC-1. Third, removal of the entire LBD did not cause significant change in activation of ER β by SRC-1 in the absence of ligand. In contrast to ER β , the activation of unliganded ER α by SRC-1 may require an intact AF-2 domain as suggested by the lack of response to SRC-1 of an AF-2-defective ER α mutant (data not shown). Since SRC-1 has been shown to interact weakly with the A/B domain of ER α *in vitro* (Lavinsky et al., 1998; Oñate et al., 1998; Webb et al., 1998), this suggests that SRC-1-mediated transcriptional activation of unliganded ER α and β may arise from differential use of AF-1- and AF-2-integrated functions.

The identification of two phosphorylation sites within the SRC-1 interaction domain of ER β AF-1 suggested

that phosphorylation could modulate its activity. Indeed, Ser-106 and Ser-124 were shown to be phosphorylated *in vitro* by the Pro-directed MAPK, which resulted in increased interaction of ER β with SRC-1. ER β AF-1 was also shown to become hyperphosphorylated after treatment of transfected cells with EGF. More importantly, effectors such as Ras, EGF, and IGF-1, which are known to trigger the activation of the MAPK pathway, contributed to a significant enhancement in the recruitment of SRC-1 by an AF-2-defective ER β mutant in the absence of E₂ *in vivo*. Phosphorylation-mediated activator/coactivator interactions have first been shown to be essential in CBP recruitment by the nuclear factor CREB (Chrivia et al., 1993). However, phosphorylation of Ser residues in CREB can lead to opposite effects, that is, phospho-Ser-133 stabilizes whereas phospho-Ser-142 disrupts secondary structure-mediated interactions between CBP and CREB (Parker et al., 1998). Interestingly, both positive and negative regulation of nuclear receptor activity has been associated with phosphorylation of the AF-1 domain (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997; Taneja et al., 1997; Shao et al., 1998). For human ER α , Ser-118 in AF-1 was reported to be *in vitro* phosphorylated by MAPK and critical for EGF and Ras stimulation in the presence of ligand (Kato et al., 1995; Bunone et al., 1996). However, Ser-118 of ER α does not seem to be involved in the recruitment of coactivators (Webb et al., 1998). For PPAR γ , MAPK-mediated phosphorylation of the A/B domain decreases its transcriptional activity (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997), presumably by decreasing the affinity of the LBD for its ligands via interdomain communication (Shao et al., 1998). Finally, RAR α AF-1 can be activated through binding to and phosphorylation by the general transcription factor TFIID (Rochette-Egly et al., 1997). Taken together, the data presented in this manuscript, supported by an accompanying paper (Hammer et al., 1999 [this issue of *Molecular Cell*]), reveal a novel mechanism of nuclear receptor activation via AF-1 phosphorylation that involves direct coactivator recruitment.

Experimental Procedures

Expression Vectors and Transfections

Mouse ER β expression plasmid was prepared by introducing a SalI-BstEII 5' extension fragment (GenBank accession number AF067422) into the previously described pCMX-mER β (Tremblay et al., 1997). This fragment extends the open reading frame by 64 amino acid residues at the beginning of the N-terminal region, resulting in a mER β of 549 residues. ER β -S (65-549), ER α , and SRC-1 expression constructs and the reporter plasmids have been described previously (Tremblay et al., 1997). The ER β AF-2-defective L509A and various serine mutants were generated by PCR mutagenesis using *Pfu* polymerase (Stratagene). All other truncated or deletion mutants of ER β and α were constructed by amplifying the appropriate fragments by PCR and subcloning them into the expression vector pCMX or fusing them to the Gal4 DBD. Cos1 cells were maintained in phenol red-free DMEM supplemented with 10% charcoal dextran-treated serum and cotransfected with, typically, 500 ng ERETKluc reporter along with 100 ng of mouse ER β or ER α expression vectors and equal amounts of human SRC-1 expression plasmid unless otherwise stated. Following transfection, cells were refed with the appropriate growth medium for 16 hr and harvested for luciferase and β -gal determination. Luciferase values have been normalized by β -gal activities and are expressed as relative luciferase units (RLU). OHT and EM-652 were obtained as previously described (Tremblay et al., 1998).

Immunoprecipitation and GST Pull-down Experiments

For immunoprecipitation, transfected 293 T cells were harvested in TEB lysis buffer (50 mM HEPES [pH 7.5], 0.4 M KCl, 0.2 mM EDTA, 0.1% NP-40) supplemented with 5 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml leupeptin. Cell lysates were precleared before incubation with ERβ antibody (clone Y-18, Santa Cruz Biotech) and protein A-Sepharose (Pharmacia) at 4°C overnight. The immunocomplex was pelleted, washed in TEB, and resolved by SDS-PAGE. Labeled proteins were visualized by autoradiography. For immunoblotting experiments, 293 T cell lysates were subjected to immunoprecipitation as above except that an antibody to HA peptide was used to recognize the HA-tagged SRC-1 expressed in transfected cells. The immunoprecipitates were analyzed by Western blot using the ERβ antibody followed by peroxidase-conjugated anti-goat IgG, and signals were visualized with the POD chemiluminescence kit (Boehringer-Mannheim). Pull-down experiments were performed with *in vitro* translated and ³⁵S-labeled SRC-1 incubated in IPAB buffer (Tremblay et al., 1997) containing 0.1% CHAPS with GST or GST fusions containing the wild-type or mutated A/B domain of ERβ immobilized on glutathione beads. Labeled proteins retained on the beads after washing were analyzed by SDS-PAGE and autoradiography.

In Vitro and In Vivo Phosphorylation

For *in vitro* phosphorylation assay, bacterially expressed and purified GST fusions of wild-type and serine-mutated ERβ (aa 96–130) immobilized on glutathione beads were resuspended in kinase buffer containing [γ-³²P]ATP and Erk2 (New England Biolabs) and incubated at 30°C for 30 min. ³²P incorporation was determined following SDS-PAGE. Gels were stained with Coomassie blue to monitor for equal loading and autoradiographed. For *in vivo* phosphorylation, subconfluent 293 T cells were transfected with wild-type or S106,124A-mutated ABCDβ (aa 65–274) expression plasmids and refed with DMEM growth medium. After 48 hr, transfected cells were metabolically labeled with [³²P] orthophosphate and treated for 20 min with EGF or PD98059, or left untreated. Cells were then harvested and subjected to immunoprecipitation with an antibody to ERβ followed by SDS-PAGE and autoradiography.

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References

Adams, M., Reginato, M.J., Shao, D., Lazar, M.A., and Chatterjee, V.K. (1997). Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J. Biol. Chem.* **272**, 5128–5132.

Aronica, S.M., and Katzenellenbogen, B.S. (1993). Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol. Endocrinol.* **7**, 743–752.

Berry, M., Metzger, D., and Chambon, P. (1990). Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J.* **9**, 2811–2818.

Bunone, G., Briand, P.-A., Miksicsek, R.J., and Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* **15**, 2174–2183.

Camp, H.S., and Tafuri, S.R. (1997). Regulation of peroxisome proliferator-activated receptor γ activity by mitogen-activated protein kinase. *J. Biol. Chem.* **272**, 10811–10816.

Chrivia, J.C., Kwok, R.P., Lamb, N., Hagiwara, M., Montminy, M.R., and Goodman, R.H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855–859.

Glass, C.K., Rose, D.W., and Rosenfeld, M.G. (1997). Nuclear receptor coactivators. *Curr. Opin. Cell Biol.* **9**, 222–232.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P., and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erbA. *Nature* **320**, 134–139.

Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**, 1150–1154.

Hammer, G.D., Krylova, I., Zhang, Y., Darimont, B.D., Simpson, K., Weigel, N.L., and Ingraham, H.A. (1999). Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Mol. Cell* **3**, this issue, 521–526.

Henttu, P.M., Kalkhoven, E., and Parker, M.G. (1997). AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors. *Mol. Cell Biol.* **17**, 1832–1839.

Hu, E., Kim, J.B., Sarraf, P., and Spiegelman, B.M. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science* **274**, 2100–2103.

Jeyakumar, M., Tanen, M.R., and Bagchi, M.K. (1997). Analysis of the functional role of steroid receptor coactivator-1 in ligand-induced transactivation by thyroid hormone receptor. *Mol. Endocrinol.* **11**, 755–767.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., et al. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**, 1491–1494.

Krstic, M.D., Rogatsky, I., Yamamoto, K.R., and Garabedian, M.J. (1997). Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor. *Mol. Cell Biol.* **17**, 3947–3954.

Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-Å. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93**, 5925–5930.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R., and Chambon, P. (1987). Functional domains of the human estrogen receptor. *Cell* **51**, 941–951.

Lavinsky, R.M., Jepsen, K., Heinzl, T., Torchia, J., Mullen, T.M., Schiff, R., Del-Rio, A.L., Ricote, M., Ngo, S., Gemsch, J., et al. (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc. Natl. Acad. Sci. USA* **95**, 2920–2925.

Metzger, D., Ali, S., Bornert, J.M., and Chambon, P. (1995). Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. *J. Biol. Chem.* **270**, 9535–9542.

Mosselman, S., Polman, J., and Dijkema, R. (1996). ERβ: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392**, 49–53.

Onate, S.A., Tsai, S.Y., Tsai, M.-J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357.

Onate, S.A., Boonyaratankornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P., and O'Malley, B.W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.* **273**, 12101–12108.

Parker, D., Jhala, U.S., Radhakrishnan, I., Yaffe, M.B., Reyes, C., Schulman, A.I., Cantley, L.C., Wright, P.E., and Montminy, M. (1998). Analysis of an activator-coactivator complex reveals an essential role for secondary structure in transcriptional activation. *Mol. Cell* **2**, 353–359.

- Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J.-M., and Chambon, P. (1997). Stimulation of RAR α activation function AF-1 through binding to the general transcription factor TFIID and phosphorylation by CDK7. *Cell* *90*, 97–107.
- Shao, D., Rangwala, S.M., Bailey, S.T., Krakow, S.L., Reginato, M.J., and Lazar, M.A. (1998). Interdomain communication regulating ligand binding by PPAR- γ . *Nature* *396*, 377–380.
- Takeshita, A., Yen, P.M., Misiti, S., Cardona, G.R., Liu, Y., and Chin, W.W. (1996). Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. *Endocrinology* *137*, 3594–3597.
- Taneja, R., Rochette-Egly, C., Plassat, J.-L., Penna, L., Gaub, M.-P., and Chambon, P. (1997). Phosphorylation of activation functions AF-1 and AF-2 of RAR α and RAR γ is indispensable for differentiation of F9 cells upon retinoic acid and cAMP treatment. *EMBO J.* *16*, 6452–6465.
- Tremblay, G.B., Tremblay, A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Labrie, F., and Giguère, V. (1997). Cloning, chromosomal localization and functional analysis of the murine estrogen receptor β . *Mol. Endocrinol.* *11*, 353–365.
- Tremblay, A., Tremblay, G.B., Labrie, C., Labrie, F., and Giguère, V. (1998). EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors α and β . *Endocrinology* *139*, 111–118.
- Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.-M., Subramanian, S., McInerney, E.M., et al. (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Mol. Endocrinol.* *12*, 1605–1618.
- Weigel, N.L., and Zhang, Y. (1998). Ligand-independent activation of steroid hormone receptors. *J. Mol. Med.* *76*, 469–479.
- Zhang, Y., Beck, C.A., Poletti, A., Clement IV, J.P., Prendergast, P., Yip, T.-T., Hutchens, T.W., Edwards, D.P., and Weigel, N.L. (1997). Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. *Mol. Endocrinol.* *11*, 823–832.