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Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator

(activation domain/nuclear receptor/coactivator/transcriptional synergism)

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ABSTRACT **The estrogen receptor (ER), a member of a large superfamily of nuclear hormone receptors, is a ligand-inducible transcription factor that regulates the expression of estrogen-responsive genes. The ER, in common with other members of this superfamily, contains two transcription activation functions (AFs)—one located in the amino-terminal region (AF-1) and the second located in the carboxyl-terminal region (AF-2). In most cell contexts, the synergistic activity of AF-1 and AF-2 is required for full estradiol (E_2)-stimulated activity. We have previously shown that a ligand-dependent interaction between the two AF-containing regions of ER was promoted by E_2 and the antiestrogen *trans*-hydroxytamoxifen (TOT). This interaction, however, was transcriptionally productive only in the presence of E_2. To explore a possible role of steroid receptor coactivators in transcriptional synergism between AF-1 and AF-2, we expressed the amino terminal (AF-1-containing) and carboxyl-terminal (AF-2-containing) regions of ER as separate polypeptides in mammalian cells, along with the steroid receptor coactivator-1 protein (SRC-1). We demonstrate that SRC-1, which has been shown to significantly increase ER transcriptional activity, enhanced the interaction, mediated by either E_2 or TOT, between the AF-1-containing and AF-2-containing regions of the ER. However, this enhanced interaction resulted in increased transcriptional effectiveness only with E_2 and not with TOT, consistent with the effects of SRC-1 on the full-length receptor. Our results suggest that after ligand binding, SRC-1 may act, in part, as an adapter protein that promotes the integration of amino- and carboxyl-terminal receptor functions, allowing for full receptor activation. Potentially, SRC-1 may be capable of enhancing the transcriptional activity of related nuclear receptor superfamily members by facilitating the productive association of the two AF-containing regions in these receptors.**

The estrogen receptor (ER) is a 66-kDa, ligand-inducible transcription factor that regulates the transcription of estrogen-responsive genes (for reviews see refs. 1–3). Like other steroid hormone receptors, the ER is a modular protein that can be divided into separable domains with specific functions, such as ligand binding, dimerization, DNA binding, and transactivation (4–7). In addition to a centrally located C domain, corresponding to the DNA binding domain, the ER contains two distinct activation functions (AFs; refs. 6–9). The AF located in the amino-terminal A/B domain is termed AF-1, and a second, hormone-dependent AF (AF-2) is located in the E domain along with the hormone binding function of ER. AF-1 and AF-2 function in a synergistic manner and are required for full ER activity in most cell contexts (7, 10, 11). Like other activation domains, the AFs of ER are thought to be important targets for basal transcriptional factors or specific cellular proteins that function as coactivators. The activity of each AF of ER varies in different cellular contexts, and these AFs have been shown to have squelching effects on their own activity and on the activity of other receptors (9), providing evidence that AF-1 and AF-2 interact with cellular proteins, which may be distinct from the basal transcription factors.

Previously, we have shown that when the amino-terminal region (ABCD) and the carboxyl-terminal region (EF) of the ER were expressed as separate polypeptides in mammalian cells, they were capable of interacting in an estradiol (E_2)-dependent manner to reconstitute the transcriptional activity of ER (12). Furthermore, we demonstrated that the interaction between ABCD and EF was also promoted by the antiestrogen *trans*-hydroxytamoxifen (TOT); however, this interaction was not transcriptionally productive. Although these studies provided information regarding ER transactivation through synergism between the two ER AFs, these studies were unable to determine whether the interaction between the amino- and carboxyl-terminal regions was direct or indirect, perhaps requiring intermediary proteins to promote the association of the AF-1- and AF-2-containing regions of the receptor. It is possible that the interaction between AF-1 and AF-2 requires accessory proteins, possibly a coactivator, to contribute to the transcriptionally productive association between the amino- and carboxyl-terminal regions of ER. We were interested in determining how coactivators, required for optimal ER transactivation, enhance receptor activity.

Using a yeast two-hybrid system, Oñate *et al.* (13) recently identified the steroid receptor coactivator-1 (SRC-1) protein, which interacted in a ligand-dependent manner with the hormone binding domain of the progesterone receptor. More recently, SRC-1 has been postulated to exist as a family of proteins related to p160 (ERAP160) (14, 15). SRC-1 was shown to significantly enhance the transcriptional activity of ER and other steroid hormone receptors. Overexpression of SRC-1 also reversed the squelching of progesterone receptor transcriptional activity upon coexpression of ligand-bound ER, demonstrating that SRC-1 is a genuine coactivator for steroid hormone receptors. It is unknown what precise function SRC-1 or other coactivators perform after binding to the receptor to result in enhanced transcriptional activity. In these studies, we use SRC-1, a coactivator for steroid hormone receptors, and examine its ability to enhance the ligand-dependent interaction of the amino- and carboxyl-terminal regions of ER, resulting in a more potent transcriptional response to estrogen.

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Abbreviations: ER, estrogen receptor; AF, activation function; E_2, estradiol; TOT, *trans*-hydroxytamoxifen; SRC-1, steroid receptor coactivator-1; ERE, estrogen response element; CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase.

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MATERIALS AND METHODS

Chemicals and Materials. Cell culture media were purchased from GIBCO. Calf serum was from HyClone and fetal calf serum was from Sigma. ^{14}C-Chloramphenicol (50–60 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/NEN.

Plasmids. The ER expression vectors (pCMV5-hER) for full-length wild-type human ER (amino acids 1–595) and ER derivatives ABCD (amino acids 1–378), M109 (amino acids 109–595), M109CD (amino acids 109–378), EF (amino acids 312–595), and EF-VP16, were constructed as described (12). An expression vector encoding SRC-1 and an empty expression vector that lacks the SRC-1 cDNA have been described (13). ER–VP16 and M109–VP16 were generated by replacing the *Bsm*I/*Bam*HI fragment of pCMV-hER or pCMV-hER(M109), respectively, with a PCR-generated fragment encoding 78 aa of the VP16 activation domain containing *Bsm*I/*Bam*HI sites. The estrogen response element (ERE)-containing reporter plasmids were $(ERE)_3$-pS2-CAT, constructed as described (12), and $(ERE)_4$-TATA-CAT, which was provided by David J. Shapiro of the University of Illinois. Either the plasmid pCH110 (Pharmacia) or pCMVβ (Clontech), which contains the β-galactosidase gene, was used as an internal control for transfection efficiency. pTZ19R carrier DNA was from Pharmacia.

Cell Culture and Transient Transfections. Chinese hamster ovary (CHO) cells were maintained and transfected as described (16). Cells were transiently transfected by $CaPO_4$ coprecipitation method and were given 400 μl of precipitate containing the following: either 10 ng of wild-type ER, ER–VP16, M109, or M109–VP16 or 500 ng of each ER-derivative expression vector; 2.0 μg of $(ERE)_4$-TATA-CAT reporter plasmid; 0.3 μg of pCH110 internal control plasmid; up to 6.0 μg of SRC-1 expression vector or empty vector; and pTZ19R carrier DNA to a total of 10 μg of DNA. After 12–16 h, cells were shocked with 20% glycerol/Hanks' balanced salt solution (HBSS) for 1.5 min, rinsed with HBSS, and given fresh medium and hormone treatment as indicated. 3T3 mouse fibroblast cells were maintained and transfected as described (12, 17). Cells were harvested 24 h after glycerol shock and hormone treatment, and extracts were prepared in 200 μl of 250 mM Tris·HCl (pH 7.5) using three freeze-thaw cycles. β-Galactosidase activity was measured to normalize for transfection efficiency and chloramphenicol acetyltransferase (CAT) assays were performed as described (16).

RESULTS

The present study was designed to aid in understanding how SRC-1 increases transcriptional activity of the ER and to determine if this involved enhancing the integration of activities of the two AFs of the receptor located in the amino- and carboxyl-terminal regions. The schematic in Fig. 1 shows the ER derivatives used in our studies. We first tested the effect of exogenous SRC-1 on the transcriptional activity of the full-length receptor in ER-negative CHO cells (Fig. 2). When expressed in cells in the absence of added SRC-1, the wild-type ER was able to induce transactivation of an ERE-containing CAT reporter gene ≈12-fold in the presence of E_2. No transcriptional activation was observed with the wild-type ER upon treatment with the antiestrogen TOT. When SRC-1 was expressed alone in cells in the absence of ER, it was unable to evoke transcription in the presence or absence of any hormone treatment tested. However, when SRC-1 was coexpressed in increasing amounts along with wild-type ER, it enhanced transcriptional activity nearly 5-fold in the presence of E_2. No transcriptional activity was observed with TOT treatment even with high levels of SRC-1. In addition, enhancement of E_2-occupied wild-type ER transcriptional activity was due to SRC-1 and not to other elements in the plasmid, as there was

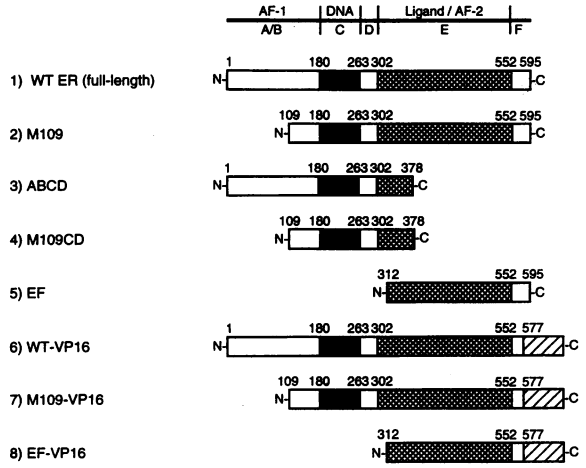

FIG. 1. Structure of ER derivatives used in this study. The structural domains of ER (A/B, C, D, E, and F), as well as the AF-1, AF-2, DNA-binding (solid boxes) and ligand-binding (cross-hatched boxes) functional domains, are shown above the schematics for the receptors. Hatched boxes represent the VP16 activation domain (residues 413–490).

no change in E_2-stimulated activity of wild-type ER when cotransfections used an empty expression vector lacking the SRC-1 cDNA (data not shown). The enhancement of E_2-dependent transcriptional activity of the ER with increasing amounts of SRC-1 implies that SRC-1 is a coactivator for E_2-dependent activity of ER, consistent with previous studies conducted in HeLa cells (13).

We then tested the ability of SRC-1 to enhance the transcriptionally productive interaction between the AF-1-containing, DNA-binding (ABCD) and the AF-2-containing, hormone-binding (EF) regions of ER (Fig. 3). Coexpression of SRC-1 with either ABCD or EF alone did not stimulate transcription of the reporter gene. When the ABCD and EF polypeptides were coexpressed in CHO cells in the absence of

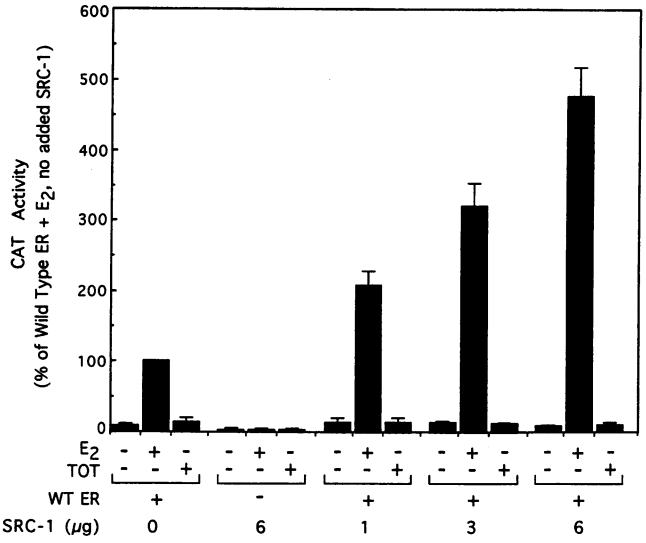

FIG. 2. Enhancement of wild-type ER transcriptional activity by SRC-1. ER-negative CHO cells were transfected with expression vectors for wild-type (WT) ER and SRC-1 as indicated, an internal control β-galactosidase plasmid, and an ERE-TATA-CAT reporter. Cells were treated with control (0.1% ethanol) vehicle, 10 nM E_2, or 1 μM TOT for 24 h. CAT activity was normalized for β-galactosidase activity from an internal control plasmid and analyzed. The CAT activity observed with wild-type ER plus E_2 but no added SRC-1 is set at 100%. Error bars represent the mean ± SEM for three or more determinations. Some error bars are too small to be visible.

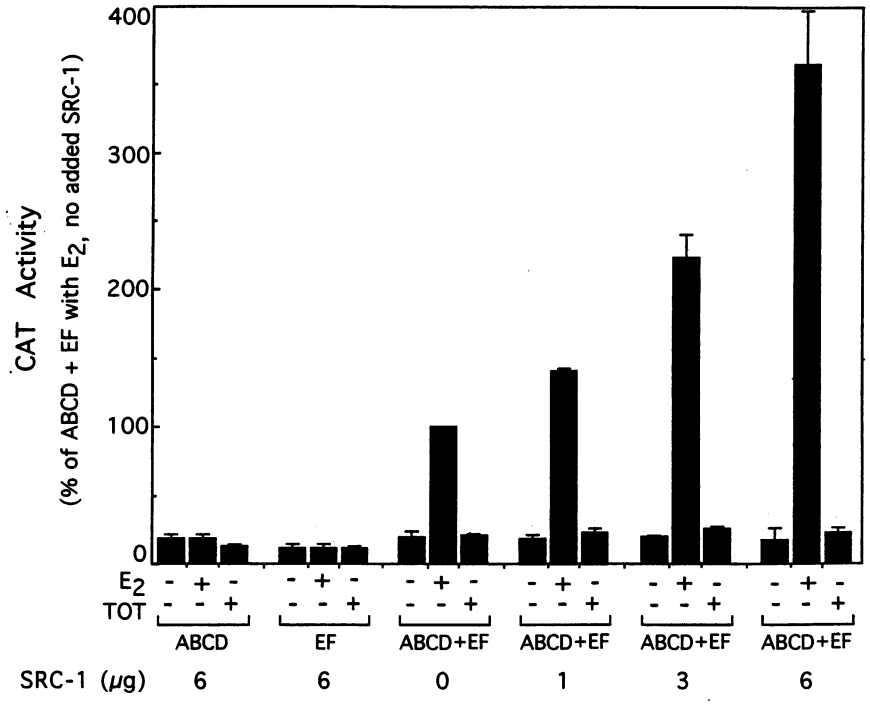

FIG. 3. E_2-dependent enhancement of the transcriptional activity of the amino- and carboxyl-terminal regions of ER by SRC-1. CHO cells were transfected with expression vectors for ER derivatives ABCD, EF, and SRC-1, as indicated, and an ERE-TATA-CAT reporter. Cells were treated with control vehicle, 10 nM E_2, or 1 μM TOT, and CAT activity, normalized for internal control β-galactosidase activity, was analyzed as described in the legend to Fig. 2.

added SRC-1, they were capable of interacting in a transcriptionally productive manner only in the presence of E_2, reconstituting ≈30% of the full-length receptor activity. When SRC-1 was coexpressed in increasing amounts with ABCD and EF, it enhanced the E_2-dependent, transcriptionally productive interaction without inducing any transcription in the absence of hormone or in the presence of TOT. These results show that coexpression of SRC-1 results in a significant increase in the transcriptional activity generated by the assembly of ABCD and EF in the presence of E_2 and not TOT, similar to the effects of SRC-1 on the full-length receptor seen in Fig. 2.

To determine if SRC-1 enhances integration of the transactivating functions of the amino- and carboxyl-terminal regions of ER, we coexpressed SRC-1 with ABCD and EF-VP16. The EF-VP16 fusion protein contains domains E and F of the human ER linked to the activation domain of the viral protein 16 (18). The constitutively active VP16 activation domain allows the detection of an interaction between ABCD and EF, even if the interaction is not transcriptionally productive. As shown in Fig. 4, coexpression of SRC-1 with either ABCD or EF-VP16 did not result in any significant transcriptional activity. When ABCD and EF-VP16 were expressed together in cells, stimulation of transcriptional activity was observed upon treatment with E_2 and to a lesser extent, TOT, indicating an interaction between ABCD and EF-VP16 in the presence of E_2 and TOT. However, when SRC-1 was coexpressed with ABCD and EF-VP16, the activity in the presence of E_2 and TOT was enhanced to ≈7-fold and ≈5-fold, respectively, above that in the absence of added SRC-1, and the enhancement occurred in an SRC-1 dose-dependent manner. In addition, when an amino-terminally truncated version of ABCD (M109CD), which lacks most of the A/B domain (i.e., lacks the first 108 aa of the receptor), was used in place of ABCD, it was unable to associate with EF-VP16 even at high levels of SRC-1, indicating that SRC-1 enhancement of ABCD and EF-VP16 activity requires an intact AF-1 region.

Similar results were obtained in the ER-negative 3T3 mouse fibroblast cell line using a different ERE-containing reporter (3ERE-pS2-CAT), where the association of the amino- and carboxyl-terminal regions of ER was enhanced ≈3-fold in the presence of E_2 or TOT with 3 or 6 μg of SRC-1 (data not presented). The magnitude of enhancement was less in the 3T3 cells compared with the CHO cells, possibly indicating higher levels of endogenous SRC-1 in the 3T3 cells.

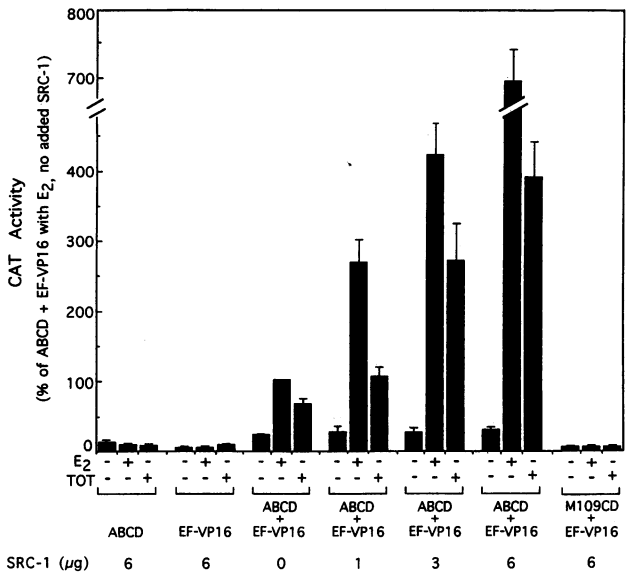

FIG. 4. Enhancement of the interaction of the amino- and carboxyl-terminal regions of ER by SRC-1. CHO cells were transfected with expression vectors for ER derivatives ABCD, M109CD, EF-VP16, and SRC-1, as indicated, and an ERE-TATA-CAT reporter. Cells were treated with control vehicle, 10 nM E_2, or 1 μM TOT, and CAT activity, normalized for internal control β-galactosidase activity, was measured as described in the legend to Fig. 2.

We also compared the effect of SRC-1 on transcriptional activity of the full-length ER or the full-length ER linked to the VP16 activation domain (ER–VP16) in the presence of E_2 or TOT. As expected, the E_2-dependent transcriptional activity of wild-type ER was enhanced by the coexpression of SRC-1 (Fig. 5*A Left*). In contrast to the wild-type ER, ER–VP16 alone stimulated substantial transcription in the absence of hormone (Fig. 5*A Right*), and this transcriptional activity was not enhanced by coexpression of SRC-1. With E_2 in the absence of added SRC-1, ER–VP16 activity was twice that seen with no hormone addition, indicating that ER–VP16 is brought more effectively to the DNA when it is liganded. SRC-1 enhanced ER–VP16 transcriptional activity in the presence of E_2, and the ≈4-fold enhancement by SRC-1 was similar in magnitude to that seen with the E_2-occupied wild-type ER. These results suggest that the increased transcription by ER–VP16 with E_2 is likely due to transcriptional enhancement of ER AF-1/AF-2 activity by SRC-1. In the presence of TOT, no transcriptional enhancement was observed when ER–VP16 was coexpressed with SRC-1. Since there is no transcription by AF-1 and AF-2 in the presence of TOT, it is perhaps not surprising that SRC-1 does not affect ER–VP16 liganded with TOT. Together, these results indicate that in this cellular context, an E_2–ER complex is needed for SRC-1 enhancement, and the VP16 activation domain was not significantly affected by SRC-1. The lack of enhancement of the VP16 activation domain by SRC-1 was not likely due to competition for limiting cellular factors required for transcription, as similar results were obtained using significantly lower (i.e., 10- or 20-fold lower) levels of ER–VP16 expression plasmid (data not shown).

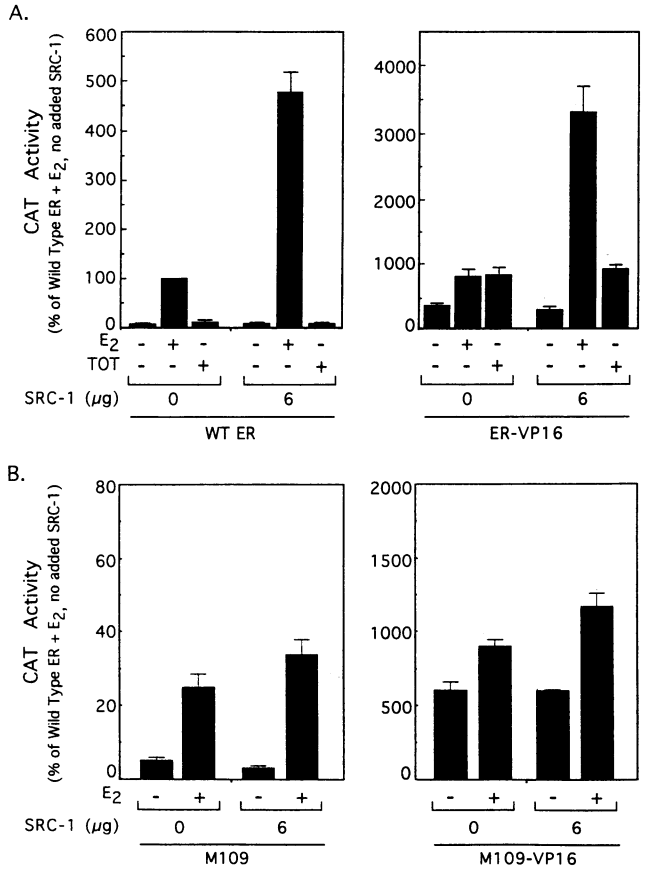

FIG. 5. Effects of SRC-1 on ER–VP16 fusion proteins. CHO cells were transfected with expression vectors for (*A*) wild-type ER or ER–VP16 or (*B*) M109 or M109–VP16, SRC-1, as indicated, and an ERE-TATA-CAT reporter. Cells were treated with control vehicle, 10 nM E_2, or 1 μM TOT, and CAT activity was analyzed as described in the legend to Fig. 2.

In related studies, we used the ER mutant, M109, which lacks most (the first 108 aa) of the A/B domain. M109 was transcriptionally impaired compared with the wild-type ER, stimulating only ≈30% of wild-type ER activity in the presence of E_2 (Fig. 5*B Left*). Upon coexpression of SRC-1, there was minimal change in the E_2-dependent transcriptional activity of M109. Similar results were obtained with M109–VP16 (Fig. 5*B Right*) in that there was little enhancement of E_2-dependent transcription upon coexpression of SRC-1. Therefore, in this cell system, deletion of AF-1 nearly fully abolished the enhancement of receptor activity by SRC-1 with both M109 and M109–VP16 in the presence of E_2. Presumably, SRC-1 still interacts with these A/B deletion receptors through the intact AF-2 region; however, the transcriptional enhancement of ER by SRC-1 requires an intact AF-1 containing A/B domain.

DISCUSSION

Our results provide one potential mechanism by which coactivators promote the full transcriptional activity of ER. The enhancement of a transcriptionally productive association of the amino- and carboxyl-terminal regions of ER through the influence of SRC-1 may be an essential step in activated transcription by hormone-occupied ER. Because of the complexity of receptor-mediated transcription, the detailed events that lead to hormone-dependent transactivation are not yet well understood. However, it is known that, after hormone binding, the ER undergoes a conformational change that is thought to allow the displacement of repressor proteins associated with the ER and to make the receptor accessible for interaction with coactivators (19, 20). The activated receptor has been postulated to aid in the stabilization of the preinitiation complex (3, 20, 21) and to play a role in the alteration of chromatin structure (1–3, 22). Our studies investigate two important aspects leading to ER-mediated transcription—namely, the conformational change in ER that is induced by ligand binding and the interaction of ER with coactivators. In this report, we have demonstrated that the ligand-induced conformational change promotes the interaction between the amino- and carboxyl-terminal regions of ER, when expressed as separate polypeptides in cells, and that this interaction is facilitated by the coactivator SRC-1. The next step, enhancement of transcriptional activity by SRC-1, requires that the ER be liganded with hormone (E_2), and not antihormone (TOT), for the integrated functions of the AF-1- and AF-2-containing regions of the ER to be transcriptionally productive. These results help in providing a clearer picture of the molecular events that occur after ligand binding to result in an activated receptor.

SRC-1 was first isolated through its ability to bind to the AF-2-containing, ligand-binding domain of progesterone receptor (13). Our results suggest that SRC-1 can act, at least in part, to functionally enhance ER activity by promoting the association between the amino- and carboxyl-terminal regions of ER. SRC-1 did not stimulate TOT-dependent wild-type ER activity and did not promote the transcriptionally productive assembly of ABCD and EF in the presence of TOT, because AF-2 is not functional when liganded with TOT (7, 23). However, SRC-1 did evoke increased activity measured with ABCD and EF-VP16 in the presence of TOT (Fig. 4 versus Fig. 3), indicating that SRC-1 promotes the functional interaction of ABCD and EF-VP16. The absence of SRC-1 stimulation of full-length ER activity when occupied with TOT highlights the important role of ligand character in the response of the receptor to SRC-1. In the cellular contexts examined, SRC-1 enhanced transcriptional effectiveness only of the E_2–AF-1/AF-2 complex, perhaps by facilitating the interaction of the two AF-containing regions of the receptor with the basal transcription complex.

Multiple proteins have been identified which interact with ER in a ligand-dependent manner (15, 24–27); however, most have not yet been shown to enhance ER-stimulated transcription. An exception is the cAMP response element-binding protein (CREB) coactivator, CREB-binding protein (CBP), another recently reported coactivator for the steroid receptor superfamily (14). SRC-1 has been shown to significantly increase the transcriptional activity of progesterone receptor and other steroid hormone receptors, including ER. Potentially, SRC-1 may function to enhance the transcription of other members of the steroid hormone receptor superfamily by a mechanism analogous to our findings. The conservation of an amino- and a carboxyl-terminal activation domain among steroid hormone receptors (2, 3) and the ability of SRC-1 to act as a coactivator for several steroid hormone receptors together suggest a general mechanism for coactivator action on steroid hormone receptors that may involve facilitation of the productive association of the two AF containing regions of these receptors, enabling optimal stimulation of transcription. At present, however, we do not have evidence that the functional interaction of AF-1 and AF-2 promoted by SRC-1 is direct. In fact, the receptor complex appears to include at least SRC-1 and CBP, and the complexity is likely to grow with the verification of functional interactions of other receptor binding proteins. Any one of these molecules could interact with the receptor, directly or indirectly, to promote the cooperative actions of AF-1 and AF-2. Continued investigation of steroid hormone receptor–coactivator complexes and their interaction with the transcription apparatus should aid in elucidating further aspects of the detailed biochemical mechanism of activated transcription.

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