Coactivator and Corepressor Regulation of the Agonist/ Antagonist Activity of the Mixed Antiestrogen, 4-Hydroxytamoxifen

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Mixed antiestrogens, such as 4-hydroxytamoxifen (4HT), act as either partial agonists or antagonists of estrogen receptor (ER) function in a tissue-, cell-, and promoter-specific manner, suggesting that intracellular factors modulate their ability to regulate transcription. To determine whether coactivators and corepressors have the capacity to modulate the relative agonist/antagonist activity of 4HT, ER-dependent gene expression was measured in the absence or presence of expression vectors for SRC-1 (steroid receptor coactivator-1) or SMRT (silencing mediator of retinoic acid and thyroid hormone receptors). In Hep G2 cells in which 4HT is an agonist, exogenous SRC-1 enhanced estradiol (E_2)- and 4HT-stimulated transcription in a dose-dependent manner, while SMRT overexpression strongly reduced basal and 4HT-stimulated gene expression with no effect on E_2 activity. These observations were not cell- or promoter-specific inasmuch as similar results were obtained in HeLa cells under conditions in which 4HT is an antagonist. A protein-protein interaction assay indicated that the full-length ER binds to SMRT *in vitro*. To assess whether relative coactivator and corepressor expression within a given cell could modulate the balance of 4HT agonist/antagonist activity, SRC-1 and SMRT were coexpressed. SMRT overexpression blocked SRC-1 coactivation of 4HT-stimulated gene expression and preferentially inhibited 4HT agonist activity whether or not exogenous SRC-1 was present. The cumulative data in this model system indicate that the relative expression of coactivators and corepressors can modulate 4HT regulation of ER transcriptional activity and suggest they could contribute to the tissue-specific ability of mixed antiestrogens to activate or inhibit ER-mediated gene expression. (Molecular Endocrinology 11: 657–666, 1997)

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INTRODUCTION

The estrogen receptor (ER) is a member of a superfamily of transcription factors that serve as nuclear receptors for small hydrophobic ligands (1, 2). Estrogen binding to its receptor induces the ligand-binding domain to undergo a characteristic conformational change, whereupon receptor dimerizes, binds to DNA, and subsequently stimulates gene expression (3–6). Two distinct activation functions (AFs) contribute to the ER's transcriptional activity. The first, AF1, is located within the amino-terminal portion of the receptor whereas the second, hormone-dependent AF2 is located in the latter half of the molecule overlapping the ligand-binding domain (6, 7). The ability of these regions to contribute to ER transcriptional activity varies with the cell and promoter examined. In some contexts, individual activation domains are the major determinants, but in most cases AF1 and AF2 synergize with one another to stimulate ER *trans*-activation of gene expression (6, 8, 9).

In addition to hormone, the ER binds ligands that serve as antiestrogens. The pure (type II) antiestrogens, exemplified by ICI 164,384 and ICI 182,780, are unable to activate the ER in nearly all instances and efficiently antagonize ER function (10, 11). In contrast, mixed antiestrogens, such as *trans*-4-hydroxytamoxifen (4HT), inhibit ER activity in a selective manner and may even activate transcription under certain conditions (12). The latter group of antihormones engender a conformational change in the ligand-binding domain distinct from that induced by estradiol (E_2) and are thought to inhibit the activity of the hormone-dependent AF2 but not AF1 (3, 13, 14). The biocharacter (agonist *versus* antagonist activity) of mixed antiestrogens varies among different tissues, cells and promoters, but it may also deviate within a given biological context. For example, tamoxifen, the metabolic precursor of 4HT, is initially an antagonist of MCF-7 breast cancer cells grown in nude mice but eventually stimulates tumor growth via an ER-agonistic action that can be blocked by the pure antiestrogen, ICI

164,384, and is not dependent upon the host animal (15–17).

When steroid receptors are occupied with agonists, the AF2 domain is thought to serve as a focal point for interaction with coactivator proteins, which are thought to act as adapters to the general transcriptional machinery and modulators of chromatin structure (18–20). In addition, it is hypothesized that the distinct, antiestrogen-induced conformation of the ligand-binding domain does not promote AF2-coactivator interactions and thereby impedes the ability of the DNA-bound receptor to activate transcription (21). However, the ER can stimulate transcription via its AF1 domain, and in some contexts 4HT is a relatively good ER agonist (8), suggesting that a mechanism(s) must exist to enable the ER and the general transcription machinery to productively associate under these conditions. In addition, the acquisition of agonist activity by 4HT in cells in which it formerly was an antagonist suggests that the differential expression of cell-specific factors may facilitate a shift in 4HT biocharacter.

Therefore, to test whether increased expression of a positively acting factor could enhance the agonist activity of a mixed antiestrogen, we examined the ability of a coactivator for the steroid receptor superfamily, steroid receptor coactivator-1 (SRC-1; Ref. 22) to stimulate ER transcriptional activity in cell/promoter contexts where 4HT is either an agonist or antagonist of ER function. We found that SRC-1 overexpression stimulated the agonist activity of 4HT, but was unable to reverse the antagonist action of this antiestrogen. We also have demonstrated that the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) protein (23), identified as a corepressor of several members of the nuclear receptor superfamily including the thyroid hormone receptor (TR) and retinoic acid receptor (RAR), could negatively influence the ability of a mixed antiestrogen to activate ER-dependent gene expression.

RESULTS

SRC-1 Increases ER Transcriptional Activity Stimulated by 4HT

In Hep G2 (human hepatocellular carcinoma) cells, the mixed antiestrogen 4HT acts as a relatively good ER agonist (8). To determine whether coactivators could contribute to this transcriptional activity, the ability of SRC-1 to enhance the ER-dependent transcription of a synthetic target gene was examined. A human ER (hER) expression vector, pSVMT-wER, and a pC3-Luc synthetic target gene, which consists of a portion (−1807 to +58) of the promoter for the third component of human complement (C3) fused to the firefly luciferase (Luc) gene, were transiently transfected into Hep G2 cells with or without an expression vector for SRC-1. As expected, 4HT was a relatively good ER agonist in the absence of exogenous coactivator (8, 24) and, under our assay conditions, 100 nM 4HT stimulated ER transcriptional activity to an extent similar to that achieved by 10 nM E_2. Although 4HT can stimulate transcription via the AP-1 transcription factor (25), estrogen-dependent expression of the pC3-Luc target gene is mediated strictly via the ER and three imperfect estrogen response elements (EREs) located within the C3 promoter (24). When increasing amounts (0→1000 ng) of an expression vector for human SRC-1 were cotransfected into these cells, basal, estrogen- and 4HT-stimulated gene expression was increased in a dose-dependent manner (Fig. 1A). In contrast, the type II antiestrogen ICI 164,384 did not activate transcription of the C3 target gene in these cells, and exogenous SRC-1 expression had little or no effect on ER transcriptional activity in the presence of this pure antagonist.

The 5′-flanking region of the C3 gene contains binding sites for a number of transcription factors, including the CCAAT/enhancer binding protein (26, 27), and to determine whether SRC-1 enhanced transcription via the imperfect EREs located within this promoter (24) and not other transcription factor-binding sites, the expression of a modified target gene, TK-C3ER1&2-Luc, consisting of the three imperfect EREs of the C3 gene linked to the heterologous thymidine kinase promoter, was examined. Both E_2 and 4HT increased transcription of TK-C3ER1&2-Luc, and exogenous SRC-1 further enhanced gene expression 3-fold (Fig. 1B). In parallel experiments, cotransfection of an identical amount of SRC-1 expression vector increased E_2- and 4HT-stimulated expression of the intact C3 promoter (pC3-Luc) by ~5-fold (see Fig. 1A), indicating that the ability of SRC-1 to coactivate ER-dependent transcription may be influenced by promoter context.

Previous studies have demonstrated that SRC-1 (also known as p160) binds to the carboxy-terminal portion of the hER (amino acids 282–595), but not to a shorter ER mutant (Δ534) lacking the last 61 amino acids (21). This deleted region encompasses sequences required for AF2 function (28), and it has been suggested that SRC-1 may be a mediator of this estrogen-dependent activation domain (21). Introduction of three amino acid substitutions (D538A/E542A/D545A) to the ER's ligand-binding domain disrupts AF2 activity but not receptor dimerization or hormone-binding affinity (28). To determine whether an intact AF2 domain is required for SRC-1 coactivation of ER-dependent transcription, HepG2 cells were transfected with expression vectors for wild type ($pRST_7$-hER) or AF2 mutant ($pRST_7$-hER-3x) ER and the pC3-Luc target gene, and transcriptional activity was assessed in the absence and presence of exogenous SRC-1. In accordance with a previous report (8), these three-point mutations significantly decreased 4HT agonist activity but had little effect on E_2-stimulated transcription of pC3-Luc in HepG2 cells. When SRC-1 expression levels were increased, transcription of the target gene by

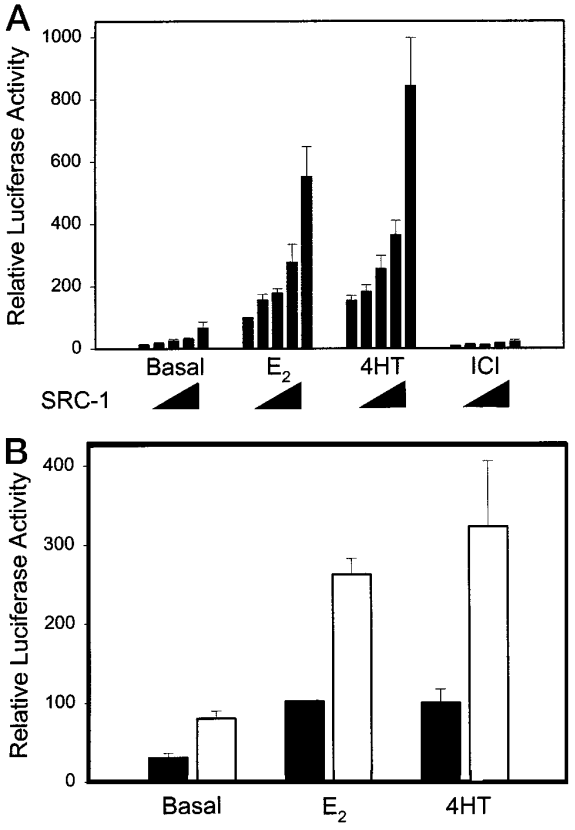

Fig. 1. SRC-1 Increases ER Transcriptional Activity Stimulated by 4HT and E_2 in Hep G2 Cells

A, Hep G2 cells were transfected with 1 μg pSVMT-wER and 2.5 μg pC3-Luc in the presence of increasing concentrations of (0, 250, 500, 750, or 1000 ng) of an expression vector (pBK-SRC-1) for SRC. The total amount of DNA transfected into each well was adjusted to 4.5 μg with pBK-CMV. B, Hep G2 cells were transfected with 1 μg pSVMT-wER and 2.5 μg TK-C3ER1&2-Luc reporter gene with 1 μg pBK-CMV (*solid bars*) or 1 μg pBK-SRC-1 (*open bars*). In both panels, estrogen-stimulated activity measured in the absence of exogenous SRC-1 was defined as 100. Data are presented as the average ± SEM of three experiments. Cells were treated with either vehicle (basal), 10 nM E_2, 100 nM 4HT, or 100 nM ICI 164,384.

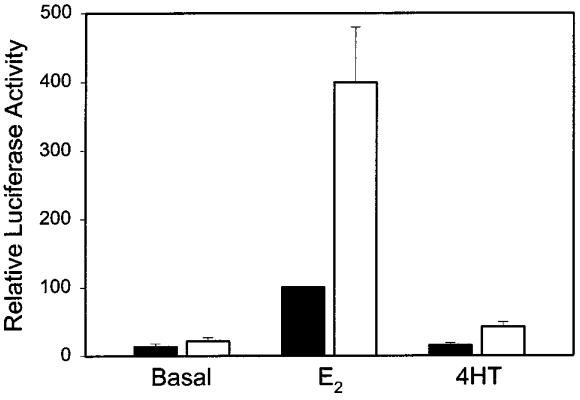

Fig. 2. SRC-1 Enhances the Transcriptional Activity of an AF2 Mutant ER

Hep G2 cells were transfected with 1 μg pRST$_7$-hER-3x and 2.5 μg pC3-Luc in the presence of 1 μg pBK-CMV (*solid bars*) or 1 μg pBK-SRC-1 (*open bars*). Estrogen-stimulated luciferase activity measured in the absence of exogenous SRC-1 was defined as 100. Data are presented as the average ± SEM of three experiments. Cells were treated with either vehicle (basal), 10 nM E_2, or 100 nM 4HT.

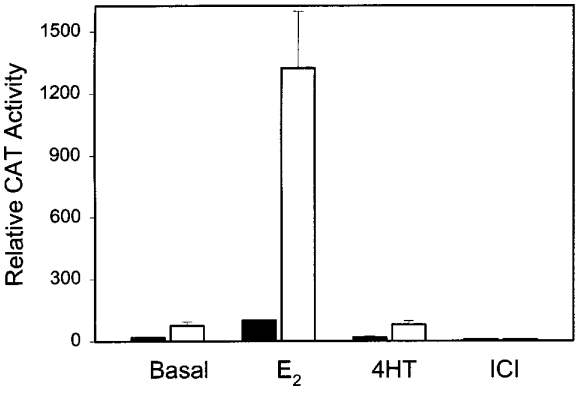

Fig. 3. SRC-1 Preferentially Increases Estrogen-Stimulated ER Transcriptional Activity in HeLa Cells

HeLa cells were transfected with 1 μg pSVMT-wER and 2.5 μg ERE-E1b-CAT in the presence of 1 μg pBK-CMV (*solid bars*) or 1 μg pBK-SRC-1 (*open bars*). CAT activity measured in cells treated with E_2 in the absence of exogenous SRC-1 was defined as 100. Data represent the average ± SEM of five experiments. Cells were treated with either vehicle (basal), 1 nM E_2, 100 nM 4HT, or 100 nM ICI 164,384 (ICI).

estrogen-activated wild type or AF2 (D538A/E542A/D545A) mutant ER was enhanced to a similar extent (Fig. 2 and data not shown) and indicates that an intact AF2 domain is not required for SRC-1 activity in this cell and promoter context.

In HeLa (human cervical carcinoma) cells, 4HT exhibits little agonist activity and effectively inhibits E_2-stimulated gene expression (29). To assess the ability of SRC-1 to contribute to ER transcriptional activity under conditions where 4HT is an antagonist, HeLa cells were transfected with a simple synthetic target gene, ERE-E1b-CAT, consisting of an ERE upstream of the E1b TATA box and the chloramphenicol acetyltransferase (CAT) gene, and a wild type hER expression vector (pSVMT-wER) with or without 1 μg of an expression vector for SRC-1. Under these assay conditions, estrogen increased CAT activity by ~6-fold, and ectopic SRC-1 expression further stimulated gene expression by 13-fold (Fig. 3). In contrast to the data obtained from Hep G2 cells, 4HT was a poor agonist in HeLa cells, and exogenous SRC-1 stimulated reporter gene expression by only ~4-fold in cells treated with vehicle or 4HT. As expected, ER transcriptional activity was very low in the presence of the pure antiestrogen ICI 164,384, whether or not exogenous SRC-1 was present.

SRC-1 Enhances Ligand-Independent Activation of ER

In the absence of exogenous ligand, the hER can be activated by dopamine receptor agonists of the D_1 subtype (29, 30) and cholera toxin/3-isobutyl-1-methylxanthine (IBMX) [agents that increase intracellular cAMP concentrations (31)]. To determine whether SRC-1 has the potential to contribute to ER transcriptional activity initiated by ligand-independent signaling pathways, HeLa cells were transfected with an ER expression vector and the ERE-E1b-CAT target gene and stimulated with forskolin (an activator of adenylate cyclase) and IBMX (a phosphodiesterase inhibitor). Elevation of intracellular cAMP levels by this treatment stimulated ER transcriptional activity by 5-fold, and ectopic SRC-1 expression further increased gene expression an additional 6-fold (Fig. 4) indicating that SRC-1 can significantly increase ER transcriptional activity in the absence of ligand when cells are appropriately stimulated. In parallel experiments, SRC-1 increased estrogen-stimulated activity by ~4-fold (Fig. 4). To ensure that forskolin/IBMX-induced transcription was ER-dependent, CAT gene expression was assessed in the presence of the pure antiestrogen ICI 164,384 with or without exogenous SRC-1, and no significant activity was observed.

As previously demonstrated (29, 32), 4HT does not antagonize ER transcriptional activity stimulated by dopamine or cAMP-signaling pathways, and the resulting gene expression is greater than that achieved by 4HT and either agent alone (Fig. 4). Since 4HT is normally an antagonist in these cells (29), these ligand-independent activation pathways also encourage a shift in the balance of this mixed antiestrogen's activity from antagonist to agonist. The ability of exogenous SRC-1 to further enhance forskolin/IBMX- stimulated ER activity in the presence of 4HT (~4-fold) suggests that this coactivator also contributes to this mechanism of gene expression.

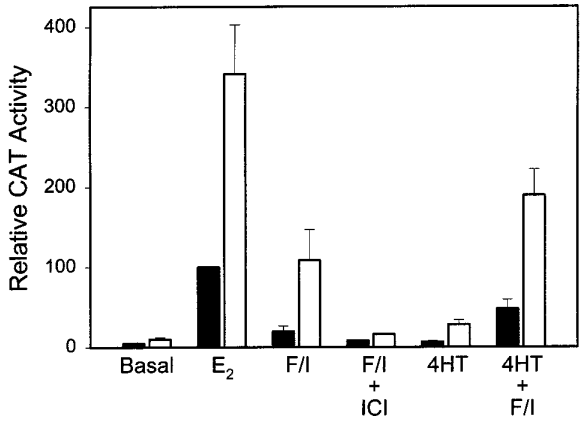

Fig. 4. SRC-1 Stimulates the Ligand-Independent Activation of ER Transcriptional Activity

HeLa cells were transfected with 1 μg pSVMT-wER and 2.5 μg ERE-E1b-CAT in the presence of 0.5 μg pBK-CMV (*solid bars*) or 0.5 μg pBK-SRC-1 (*open bars*). ER transcriptional activity determined in the presence of E_2 and absence of exogenous SRC-1 was defined as 100. Data are presented as the average ± SEM of three experiments. Cells were treated with vehicle (basal), 1 nM E_2, 5 μM forskolin and 50 μM IBMX (F/I), 100 nM ICI 164,384 (ICI), 100 nM 4HT, or combinations thereof.

The Corepressor, SMRT, Inhibits 4HT Agonist Activity

It is clear that in comparison to estrogens, the relative ability of 4HT to activate ER-dependent transcription varies between cell and tissue types (14, 24, 33), and it has been postulated that cellular coregulatory proteins contribute to the differential biocharacter of 4HT (12). Although SRC-1 stimulated 4HT agonist activity in Hep G2 cells, it was unable to convert 4HT from an antagonist to agonist in HeLa cells. Therefore, the possibility that a corepressor protein may help to define the agonist/antagonist balance of 4HT activity was examined. In HepG2 cells, where 4HT is a relatively good ER agonist, exogenous SMRT did not decrease E_2-dependent transcription (Fig. 5A). However, SMRT attenuated 4HT's agonist activity (>70%) with the result that the ability of 4HT to activate transcription was significantly attenuated in comparison to estrogen. SMRT also decreased basal ER activity by 60%. In contrast, SMRT had little effect on the already low activity of ER in the presence of the pure antiestrogen ICI 164,384. Overall changes in gene expression are unlikely to account for SMRT inhibition of 4HT-stimulated ER activity because exogenous SMRT did not influence β-galactosidase activity expressed from a SV40- or cytomegalovirus (CMV)-regulated constitutive expression vector (data not shown). When SMRT was expressed ectopically in HeLa cells, it did not inhibit estrogen-stimulated expression of the ERE-E1b-CAT reporter gene (Fig. 5B). However, it further decreased the low 4HT agonist activity observed in these cells. Taken together, these data indicate that ectopic expression of this corepressor decreases 4HT agonist activity whether it is weak (HeLa cells) or relatively strong (Hep G2 cells).

Interaction of ER and SMRT

Since SMRT altered 4HT-stimulated ER activity in transient transfection assays, the ability of ER and SMRT to physically interact was assessed *in vitro* by glutathione-*S*-transferase (GST) pull-down assay. A fusion protein consisting of GST fused to the amino terminus of full-length hER was incubated with ^{35}S-labeled, *in vitro* translated SMRT protein (amino acids 29–1495) in the absence or presence of E_2 and tamoxifen. Virtually no SMRT protein was retained by GST alone (Fig. 6). In comparison to the input lane, significant levels of SMRT were retained by GST-ER regardless of the presence of estrogen or antiestrogen. Thus,

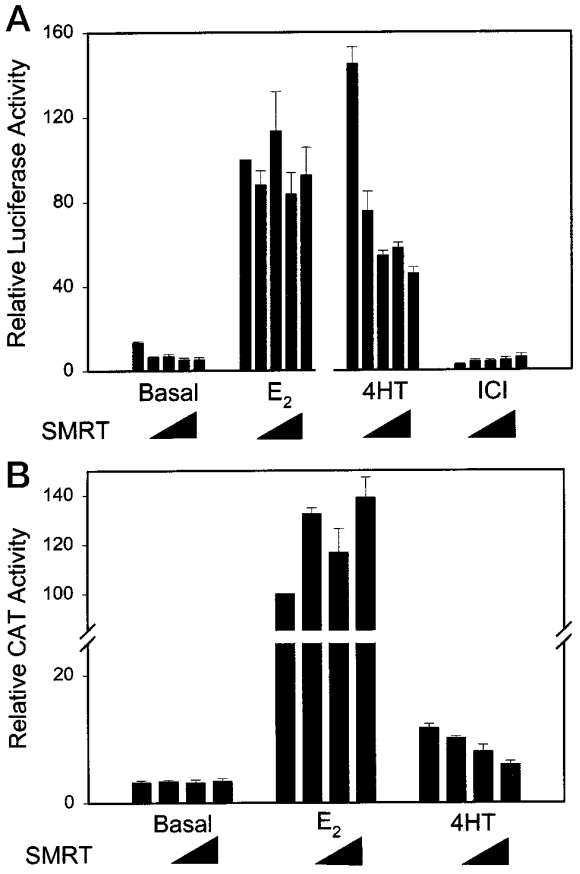

Fig. 5. SMRT Preferentially Inhibits 4HT-Stimulated ER Transcriptional Activity

A, Hep G2 cells were transfected with 1 μg pSVMT-wER and 2.5 μg pC3-Luc in the presence of increasing concentrations (0, 250, 500, 750, or 1000 ng) of an expression vector (pABΔgal-SMRT) for SMRT. Total DNA transfected into each well was adjusted to 4.5 μg with pABΔgal. Cells were treated with vehicle (basal), 10 nM E_2, 100 nM 4HT, or 100 nM ICI 164,384. B, HeLa cells were transfected with 1 μg pSVMT-wER and 2.5 μg ERE-E1b-CAT reporter gene with increasing concentrations of pABΔgal-SMRT (0, 500, 1000, or 2500 ng). The total DNA transfected into each well was adjusted to 6 μg with pABΔgal. Cells were treated with vehicle (basal), 1 nM E_2, or 100 nM 4HT. In both panels, ER transcriptional activity in the presence of E_2 and absence of exogenous SMRT were defined as 100. Data represent the average ± SEM of at least three experiments.

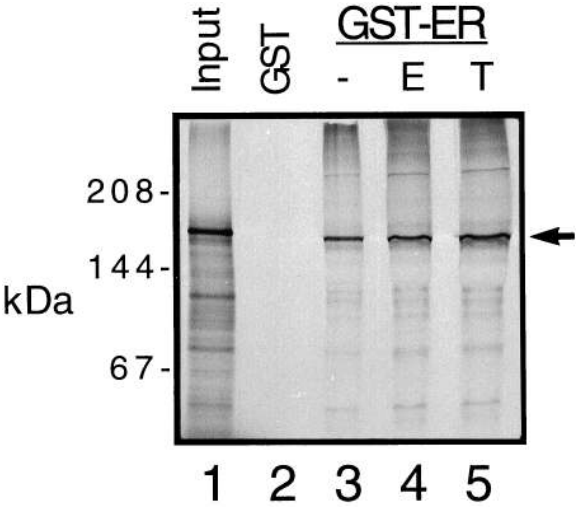

Fig. 6. *In Vitro* Interaction of GST-ER with SMRT

^{35}S-labeled SMRT (amino acids 29–1495) was tested for interaction with GST alone (lane 2) or GST-ER in the presence of vehicle (lane 3), 1 μM E_2 (lane 4), or 1 μM tamoxifen (lane 5). The *arrow* (*right*) indicates the position of SMRT. Lane 1 shows 25% of the input [^{35}S]SMRT used in these assays.

SMRT and ER interact *in vitro*, but in a hormone-independent manner.

SRC-1 Overexpression Does Not Reverse SMRT Inhibition of 4HT Agonist Activity

The above studies indicated that both SRC-1 and SMRT have the potential to modulate ER-dependent gene expression stimulated by 4HT. To determine whether perturbation of coactivator and corepressor levels within a given cell could alter 4HT-induced ER transcriptional activity, various combinations of expression vectors for SRC-1 and SMRT were introduced into HepG2 cells and target gene expression was assessed. Ectopic expression of SMRT substantially decreased 4HT-induced transcription of the pC3-Luc reporter gene in comparison to E_2 (Fig. 7; compare lanes 5 and 6 to 2 and 3). Increasing SRC-1 expression in these cells did not restore 4HT agonist activity relative to E_2, even when 50% less SMRT expression vector was cotransfected (compare lanes 11 and 12 to 5 and 6). As anticipated, in the absence of exogenous SMRT, ectopic SRC-1 increased the ER transcriptional activity induced by E_2 and 4HT equally well. Thus, within a given cell, elevated expression of SMRT shifts the balance of 4HT activity from an agonist to antagonist, and overexpression of SRC-1 was unable to reverse this process.

DISCUSSION

SRC-1 Stimulates 4HT Agonist Activity

Mixed antiestrogens, such as 4HT, regulate ER transcriptional activity in a tissue-, cell-, and promoter-dependent manner. Accumulating evidence suggests that the differential ability of partial antagonists to modify gene expression cannot be accounted for by alterations in the ligand-receptor complex alone, but also must take into consideration coregulator (coactivator and corepressor) proteins that regulate ER interactions with the general transcriptional machinery and chromatin (12). Therefore, a putative coactivator and corepressor of the steroid receptor superfamily were tested to determine whether these coregulators have

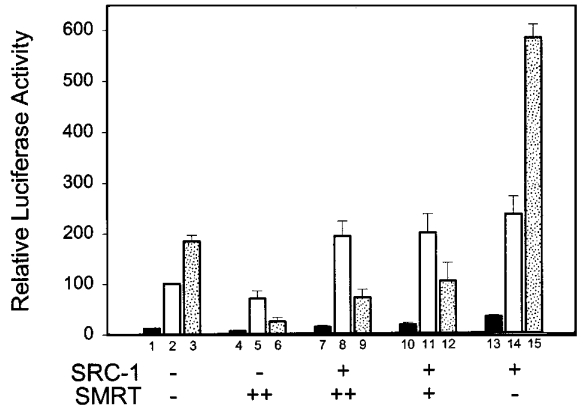

Fig. 7. Ectopic SRC-1 Expression Does Not Overcome SMRT Inhibition of 4HT Agonist Activity

Hep G2 cells were transfected with 1 μg pSVMT-wER and 2.5 μg pC3-Luc and either 0.5 μg pBK-CMV and 1 μg pABΔgal (lanes 1–3), 0.5 μg pBK-CMV and 1 μg pABΔgal-SMRT (lanes 4–6), 0.5 μg pBK-SRC-1 and 1 μg pABΔgal-SMRT (lanes 7–9), 0.5 μg pBK-SRC-1 and 0.5 μg pABΔgal-SMRT (lanes 10–12), or 0.5 μg pBK-SRC-1 and 1 μg pABΔgal (lanes 13–15). The activity of the ER in the presence of E_2 and absence of exogenous SRC-1 and SMRT was defined as 100. Data represent the average ± SEM of three experiments. Cells were treated with either vehicle (basal; *solid bars*), 10 nM E_2 (*open bars*), or 100 nM 4HT (*stippled bars*).

the potential to modify the biocharacter of a mixed antiestrogen in cell-based, ER-dependent transcription assays. Ectopic SRC-1 expression strongly enhanced estrogen-stimulated expression of two different target genes (ERE-E1b-CAT and pC3-Luc, respectively) in HeLa and Hep G2 cells and indicates that the ability of SRC-1 to enhance ER transcriptional activity is not cell or promoter restricted. In contrast, SRC-1 only weakly augmented 4HT's low agonist activity in HeLa cells, but enhanced 4HT-stimulated transcriptional activity to an extent equivalent to that of estrogen in HepG2 cells, indicating that ER liganded with either a full or partial agonist is an equivalent substrate for SRC-1 coactivation in the latter promoter and cellular context. Therefore, SRC-1 has the potential to contribute to ER transcriptional activity stimulated by estrogen or 4HT, but this coactivator is not a dominant determinant of the agonist *versus* antagonist activity of this antihormone.

AF2 Is Not Required for SRC-1 Coactivation

Steroid receptor coactivator-1 (p160) was postulated to act as a coactivator/mediator for the AF2 domain of steroid receptor superfamily members (22), and it was predicted that SRC-1 would not interact functionally with the ER when liganded with antiestrogens (21). However, the demonstration that SRC-1 enhanced 4HT-stimulated, AF1-dependent ER transcriptional activity in Hep G2 cells and efficiently coactivates the E_2-stimulated transcriptional activity of an AF2-defective ER mutant (D538A/E542A/D545A) suggests that this coactivator may act through regions in addition to AF2. In support of this, a recently published study utilizing a modified mammalian two-hybrid approach in Chinese hamster ovary cells indicated that SRC-1 facilitated the physical interaction between the amino-terminal (containing AF1) and carboxy-terminal (containing AF2) regions of ER (34). Furthermore, SRC-1 binds to an ERΔ534 mutant when examined in the context of the full-length receptor (35) instead of the ligand-binding domain alone (21). The remaining studies reporting a lack of 4HT-dependent interaction between SRC-1 (p160) and ER were conducted with the ligand-binding domain alone or in MCF-7 cells in which 4HT is typically a weak agonist or antagonist (21) and AF1 contributions to SRC-1 interaction(s) with the ER were not likely to have been assessed.

SRC-1 and Ligand-Independent Activation of the ER

The coactivation of forskolin/IBMX-stimulated ER transcriptional activity by SRC-1 in the absence of exogenous ligand suggests that a hormone-independent mechanism exists to facilitate a functional interaction of ER and this coactivator. Furthermore, the ability of ligand-independent signaling pathways to shift 4HT activity from antagonist to agonist and thereby enable SRC-1 to strongly enhance 4HT activity in a cell/promoter context where this antiestrogen is typically a relatively poor agonist, supports this hypothesis. Because all agents (*i.e.* dopamine, cAMP, growth factors) capable of ligand-independently activating the ER initiate or alter the activity of an intracellular signal transduction cascade and presumably kinases and/or phosphatases (reviewed in Ref. 36), it is possible that phosphorylation of the ER and/or SRC-1 may contribute to their ability to functionally interact with one another or with other accessory transcription factors (*e.g.* CREB binding protein; CBP) necessary for steroid receptor-dependent transcription (37). Interestingly, CBP serves as a coactivator for numerous, diverse transcription factors (*e.g.* CREB, Elk-1, c-Jun, c-Myb, c-Fos), and at least some of these interactions are dependent and/or enhanced by the phosphorylation of the site-specific activator (38–42).

SMRT Regulation of 4HT Biocharacter

Since SRC-1 was unable to alter the agonist/antagonist balance of 4HT activity, SMRT was tested for its ability to alter 4HT's agonist/antagonist activity to determine whether a corepressor has the potential to regulate mixed antiestrogen activity. In both cell/promoter contexts tested, ectopic expression of SMRT decreased the ability of 4HT to activate transcription while having little or no effect on estrogen-stimulated gene expression; this suggests that full agonists enable the ER to overcome corepressor function, while partial agonist/antagonists do not. Although the ex-

periments presented in this paper used a SMRT expression vector lacking the coding region for the first 28 amino acids, they have been repeated with another expression vector (pCMX-SMRT) that directs the expression of a SMRT isoform containing the authentic amino terminus with essentially identical results (data not shown). This indicates that the first 28 amino acids of this corepressor are not required to modulate 4HT's ability to activate ER-dependent gene expression.

The corepressor SMRT was reported to bind to TR and RAR in the absence, but not the presence, of their cognate ligands via a portion of the hinge region referred to as the CoR box (23, 43, 44). Although there is no homology between the CoR box and any region of the ER, three other structural motifs have been implicated in the association between corepressors and two orphans of the steroid receptor superfamily, Rev-ErbA and COUP-TF1; none displays significant homology to the CoR box or the ER (45–47). Thus, dissimilar receptor sequences facilitate interaction between different nuclear receptor superfamily members and SMRT. Although SMRT preferentially bound to a GST-ER fusion protein in comparison to GST alone, no hormone-dependent interaction was observed, and this could reflect differences between *in vitro* and *in vivo* binding conditions. For example, if hormone-dependent, ER-coactivator interactions contribute to the displacement/inactivation of corepressor *in vivo*, differences in ER-SMRT *in vitro* interactions are not likely to be observed. Alternatively, SMRT may interact with the hormone-independent AF1 domain of ER. Importantly, our data should not be used to implicate SMRT itself as the authentic, native corepressor for the ER. It is equally likely that another unidentified protein(s) regulates ER transcriptional activity with improved specificity and/or affinity.

Roles of Potential ER Corepressors

Although heat shock protein interactions contribute to maintaining the ER in a transcriptionally inactive state within the cell (48), under certain conditions ER is present within the nucleus and able to bind DNA in the absence of ligand (49), suggesting that another mechanism may inhibit this receptor's basal transcriptional activity. For instance, corepressors could recruit histone deacetylase activity to a target gene promoter and thereby maintain chromatin in an inactive state, directly interact with and inhibit the general transcription complex formed at the promoter (TATA), inhibit functional ER-coactivator interactions, or block the transcriptional synergism between AF1 and AF2 required for full ER activity in most cell/promoter contexts (6, 8, 9). Thus, it is postulated that the unliganded ER is bound to a corepressor(s) that either dissociates or is inactivated upon estrogen binding; the resulting conformational change would enable the ER to preferentially associate with coactivator(s) and directly and/or indirectly encourage the general transcriptional machinery to activate target gene expression. In contexts where they act as antagonists, mixed antiestrogens may induce a ligand-binding domain conformation that enables the receptor to retain its ability to interact with corepressor(s) and/or decreases its affinity for SRC-1 such that corepressors are not efficiently inactivated. Alternatively, if the mixed antiestrogen-occupied ER simultaneously binds coactivators and corepressors under these conditions, the repressor domain(s) of corepressors may inhibit ER transcriptional activity by blocking the activation function of coactivators.

The agonist activity of 4HT may manifest itself in tissues and cells where corepressor expression is low, ER transcriptional activity is stimulated by corepressor-insensitive coactivators and/or cell-specific corepressors are unable to bind to ER liganded with mixed antiestrogens. In support of the concept that an authentic ER corepressor may exist is the demonstration of a repressor domain within the ER's ligand-binding domain (amino acids 370–470) that functions independently of heat shock protein interaction (50). Furthermore, there is a precedent for the existence of a corepressor(s) for "steroid" as opposed to a orphan (Rev-ErbA) or type II (TR and RAR) receptor. A repressor domain has been mapped within the extreme carboxy terminus of the progesterone receptor, and it appears to require an additional cellular factor(s) to inhibit transcription (51).

Implications of Coregulators for 4HT Biocharacter

Collectively, these data accumulated in model transfection assays indicate that cellular coactivators and corepressors have the potential to contribute to the overall ability of a mixed antiestrogen to regulate ER target gene expression. At the tissue/organ mRNA level, the coactivators and corepressors examined to date appear to be ubiquitously expressed (22, 52–56), and presumably most, if not all, cells contain both forms of coregulators. However, the relative expression levels of known coactivators and corepressors have not been assessed, and it is likely that novel coregulators of the nuclear receptor superfamily remain to be identified. It is possible that authentic ER corepressor proteins may be expressed at relatively high levels in cells where 4HT is an antagonist and/or coactivators are present in comparatively large amounts in contexts where 4HT is an agonist. It is also likely that promoters contribute to the relative balance of 4HT agonist/antagonist activity by imposing spatial constraints on the ability of ER and coactivators/corepressors to make protein-protein interactions.

Tamoxifen, in its role as an antiestrogen, is an important endocrine therapy for the treatment of breast cancer and more recently is being tested as a chemopreventative agent in women with a high risk of developing this disease (57). However, it also exerts estrogen-like effects in bone, liver and urogenital tissues; of which some (*e.g.* an increased occurrence rate of en-

dometrial cancer) are clearly not beneficial (58). Our data highlights the necessity to understand the role that native intracellular factors play in the interpretation of tamoxifen/4HT biocharacter in cells/tissues that endogenously express ER and provides a theoretical rationale for the contribution of coregulatory proteins to the tissue-specific and gene-specific activity of ER agonists and antagonists.

MATERIALS AND METHODS

Chemicals

17β-Estradiol (E_2) and tamoxifen were obtained from Sigma Chemical Company (St. Louis, MO). The antiestrogens, ICI 164,384 and ICI 182,780, and 4HT were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively. Forskolin and IBMX were purchased from Research Biochemicals International (Natick, MA).

Plasmid DNAs

The mammalian expression vectors for full-length hER (pSVMT-wER) and the AF2 (D538A/E542A/D545A) mutant ER ($pRST_7$-hER-3x) and its corresponding wild type ER counterpart ($pRST_7$-hER) have been described previously (8, 29). The synthetic target genes, ERE-E1b-CAT (29), pC3-Luc (8), and TK-C3ER1&2-Luc (24) have been used in previous studies. Mammalian expression vectors for SMRT (pABΔgalSMRT or pCMX-SMRT) and SRC-1 (pBK-SRC-1) and the corresponding parent vectors (pABΔgal and pBK-CMV) were described previously (22, 23, 47), as was the baculoviral expression vector, pGST-hER (3).

The *in vitro* transcription vector for SMRT, pT7-SMRT(29–1495), was constructed as follows. First, human skeletal muscle poly(A)$^+$ RNA (Clontech, Palo Alto, CA) was reverse transcribed using the primer, 5′-GCTGGCATGTTCCTGCACCG-3′, and this material was used as a template cDNA for PCR amplification using the primers, 5′-AGCTGACGTCGACGCCTCGTG-3′ and 5′-CTGCACCGCCTGGCTTCTAT-3′. The resulting product was cloned into the TA cloning vector, pCR3 (Invitrogen, Carlsbad, CA) to produce pCR3-SMRT(565–1289). Subsequently, this vector was digested with *Eco*RV and *Bgl*II and ligated with the *Eco*RI (filled) to *Bgl*II fragment of pGAD10-SMRT(1192–1495) to yield the pCR3-SMRT(565–1495) vector. The cDNA insert of pGAD10-SMRT(1192–1495) was isolated from a brain cDNA library by yeast two-hybrid assay using the ligand-binding domain of the human TR as bait. The *Sal*I-*Xho*I fragment of pCR3-SMRT(565–1495) was isolated, repaired with Klenow DNA polymerase, and cloned into the *Nco*I-*Eco*RI (filled) site of the pT7βSal vector (59) to produce pT7-SMRT(565–1495). Next, human skeletal muscle was reverse transcribed using the primer, 5′-GTGCGGGACTTGGCGATCT-3′, and the resulting cDNA was amplified by PCR with the primers, 5′-AAGATTCCGAGCTCTGCTAC-3′ and 5′-CACGAGGCGTCGACGTCAGC-3′. This PCR product was TA Cloned (Invitrogen) to create pCR3-SMRT(29–564). Finally, to construct pT7-SMRT(29–1495), the *Sal*I fragment of pCR3-SMRT(29–564) was inserted into the *Sal*I site of pT7-SMRT(565–1495).

Cell Culture and Transfections

HeLa and HepG2 cells were routinely maintained in DMEM supplemented with 10% FBS. Twenty-four hours before transfection, 3×10^5 HeLa or 8×10^5 HepG2 cells were seeded per well of a six-well multiwell dish in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped serum. Cells were transfected with the indicated DNAs using Lipofectin (Life Technologies, Grand Island, NY) according to the manufacturer's guidelines. Six hours later, the DNA/Lipofectin mixture was removed and cells were fed with phenol red-free media containing 5% stripped serum and the hormone treatments indicated in the figure legends. Twenty-four hours thereafter, cells were harvested and extracts were assayed for CAT (29) or luciferase activity using the Luciferase Assay System (Promega, Madison, WI). Duplicate samples were measured in each experiment, and data are presented as the average ± SEM of at least three experiments.

Protein-Protein Interaction by GST Pull-down Assay

The full-length hER was expressed as a GST fusion protein in a baculovirus expression system in the presence of ethanol (vehicle), 1 μM estradiol, or 1 μM tamoxifen and purified using glutathione-Sepharose affinity chromatography as described previously (60). Radiolabeled SMRT (amino acids 29–1495) was produced from the pT7-SMRT(29–1495) vector with the TNT-Coupled Reticulocyte Lysate System for *in vitro* transcription and translation as recommended by the manufacturer (Promega).

To assess protein-protein interactions, equivalent levels of GST alone or GST-hER (as assessed by Coomassie staining) were incubated with glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) in NENT buffer (20 mM Tris, pH 8.0 containing 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing either ethanol, 1 μM E_2, or 1 μM tamoxifen for 60 min at room temperature. Subsequently, the supernatant was removed and the beads were washed twice with NENT buffer. Eight microliters of ^{35}S-labeled SMRT were incubated with the beads in NENT buffer containing vehicle, estrogen, or tamoxifen for 2 h at room temperature. Beads were washed five times with NENT buffer, dried, resuspended in 50 μl SDS-PAGE loading buffer, resolved by 7.5% SDS-PAGE, and visualized by fluorography.

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