Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors α and β by coactivators and corepressors

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Abstract

One mechanism by which ligand-activated estrogen receptors α and β (ERα and ERβ) stimulate gene transcription is through direct ER interaction with specific DNA sequences, estrogen response elements (EREs). ERE-bound ER recruits coactivators that stimulate gene transcription. Binding of ER to natural and synthetic EREs with different nucleotide sequences alters ER binding affinity, conformation, and transcriptional activity, indicating that the ERE sequence is an allosteric effector of ER action. Here we tested the hypothesis that alterations in ER conformation induced by binding to different ERE sequences modulates ER interaction with coactivators and corepressors. CHO-K1 cells transfected with ERα or ERβ show ERE sequence-dependent differences in the functional interaction of ERα and ERβ with coactivators steroid receptor coativator 1 (SRC-1), SRC-2 (glucocorticoid receptor interacting protein 1 (GRIP1)), SRC-3 amplified in breast cancer 1 (AIB1) and ACTR, cyclic AMP binding protein (CBP), and steroid receptor RNA activator (SRA), corepressors nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid and thyroid hormone recpetors (SMRT), and secondary coactivators coactivator associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1). We note both ligand-independent as well estradiol- and 4-hydroxytamoxifen-dependent differences in ER-coregulator activity. *In vitro* ER-ERE binding assays using receptor interaction domains of these coregulators failed to recapitulate the cell-based results, substantiating the importance of the full-length proteins in regulating ER activity. These data demonstrated that the ERE sequence impacts estradiol- and 4-hydroxytamoxifen-occupied ERα and ERβ interaction with coregulators as measured by transcriptional activity in mammalian cells.

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Introduction

Estrogens exert a wide variety of effects on growth, development, and differentiation primarily through binding to a specific intranuclear estrogen receptor protein (ER) encoded by two genes: α and β (ERα and ERβ) (Couse & Korach 1999). Stimulation of target gene expression in response to 17β-estradiol (E_2), or other agonists, is thought to be mediated by two mechanisms: (1) direct binding of E_2-liganded ER (E_2-ER) to a specific sequence called an estrogen response element (ERE) and (2) 'tethering', in which ER interacts with another DNA-bound transcription factor, e.g. Sp1 (Safe 2001) or AP-1 (Webb *et al.* 1995), in a way that stabilizes the DNA binding of that transcription factor in the absence of direct ER-DNA binding. Both processes result in recruitment of coactivators and components of the RNA polymerase II transcription initiation complex that enhances target gene transcription (Klinge 2000).

We recently reviewed studies on ER interaction with 38 estrogen-responsive genes whose promoters or 3′ untranslated regions contain functional EREs (Klinge 2001). Most estrogen-regulated genes contain imperfect, non-palindromic EREs. One conclusion from our review is that the more nucleotide changes there are from the consensus

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within a half-site of the ERE palindrome, the lower the ERα binding affinity and the lower the transcriptional activity (Klinge 2001). Further, EREs in which nucleotides are altered in both arms of the palindrome show lower transcriptional activity than those containing alterations in only one ERE half-site (Klinge 2001). These results have been confirmed experimentally (Driscoll *et al.* 1998, Tyulmenkov & Klinge 2000*a*, 2001*a,b*, Tyulmenkov *et al.* 2000, Kulakosky *et al.* 2002).

Lefstin & Yamamoto (1998) proposed that response elements recognized by nuclear transcription factors, including members of the steroid/nuclear receptor superfamily, contain information that is interpreted by bound regulator factors. Our data fit the model of Lefstin and Yamamoto by showing that different EREs bound ERα with different affinity and resulted in different amounts of transactivation of a reporter plasmid in transfected cells (Tyulmenkov *et al.* 2000, Klinge *et al.* 2001, Tyulmenkov & Klinge 2001*b*, Kulakosky *et al.* 2002). We and other investigators have proposed that DNA acts as an allosteric effector whose binding alters ER conformation such that different EREs would be hypothesized to regulate ER interaction with other proteins, e.g. coactivators or corepressors (reviewed in Klinge *et al.* 2001). In concordance with this hypothesis, we observed differences in ERα conformation, assessed by α-chymotrypsin or trypsin digestion, upon interaction with a palindromic ERE or non-palindromic EREs from the human pS2, progesterone receptor (PR), and *c-fos* genes and a direct repeat of the ERE half-site separated by 5 bp (Klinge *et al.* 2001, Ramsey & Klinge 2001). The ERE-mediated increase in ERα sensitivity to protease digestion correlated with E_2-stimulated transcription from the same EREs in transiently transfected cells and with the affinity of ERα-ERE binding *in vitro* (Klinge *et al.* 2001, Ramsey & Klinge 2001, Tyulmenkov & Klinge 2001*a,b*, Kulakosky *et al.* 2002).

Similarly, other investigators have reported differential recognition of ERα by select antibodies when ERα was bound to the *Xenopus laevis* vitellogenin A2 (vit A2) ERE versus the non-palindromic ERE from the human pS2 gene in electrophoretic mobility shift assay (EMSA) experiments (Wood *et al.* 1998). Limited protease digestion of ERα bound to vit A2 ERE or the non-palindromic pS2, vitellogenin B1, and oxytocin EREs revealed different sized [^{32}P]DNA fragments in EMSAs (Wood *et al.* 2001). These data were interpreted as indicating that ERα binding to different EREs changes ERα conformation (Wood *et al.* 1998, 2001). Likewise, experiments using phage display revealed conformational differences in ERα and ERβ when bound to vit A2, pS2, lactoferrin, and complement 3 (C3) EREs *in vitro* (Hall *et al.* 2002).

Only a few investigators have examined how these ERE-induced alterations in ER conformation impact ER-coactivator interaction. One study reported that GRIP1 showed less interaction when ERα bound to the pS2 ERE versus the vit A2, vitellogenin B1, or oxytocin EREs, indicating that the ERE sequence impacts ERα-GRIP1 interaction *in vitro* (Wood *et al.* 2001). Similar conclusions based on EMSA and GST-pulldown assays were recently reported by another group of investigators (Yi *et al.* 2002). However, the functional significance of these observations of ERE-dependent differences in ER conformation and recognition by coactivator receptor interaction domains (RIDs) has not been tested by cell-based transcription assays.

Over the past 6 years, at least 28 different ERα coactivator proteins have been identified (McKenna *et al.* 1999, Klinge 2000, McKenna & O'Malley 2002). Less information is available regarding ERβ-coactivator interaction. In brief, coactivators function to (1) acetylate the N-terminal tails of lysine residues in histones H3 and H4 leading to 'relaxed' chromatin structure (reviewed in Struhl 1998), (2) acetylate other transcription factors and coactivators (Jiang *et al.* 2000), (3) recruit secondary coactivators including coactivator associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) that methylate histones (Koh *et al.* 2001), (4) interact with components of various ATP-dependent chromatin-remodeling complexes (Hebbar & Archer 2003), and (5) direct interaction with and stabilization of basal transcription factor binding (reviewed in Hager *et al.* 1998). Once the transcription initiation complex is complete, RNA polymerase II is recruited to the transcription start site and begins transcription.

Most of the cell-based assays examining the effects of coactivators on ER-mediated transcription have used reporter genes containing two or three tandem copies of the vit A2 ERE (Norris *et al.* 1998, Lee *et al.* 1999, Mak *et al.* 1999,

Delage-Mourroux *et al.* 2000). The non-palindromic EREs that have been used in transfection experiments are from the complement C3, transforming growth factor-β3, and lactoferrin gene promoters that were used to examine the functional interaction of ERα with the receptor of estrogen receptor activity (Delage-Mourroux *et al.* 2000) and SRC-2/GRIP1 (Norris *et al.* 1998).

Selective estrogen receptor modulators (SERMS) *in vitro* and in transfected cells (Metivier *et al.* 2002). Treatment of cells with the histone deacetylase (HDAC) inhibitor trichostatin A has been shown to enhance ligand-independent as well as E_2-, tamoxifen-, and raloxifene-stimulated ERα transcription of an ERE-reporter gene, indicating that repression of ERα transcriptional activity is mediated by a corepressor-HDAC complex (Webb *et al.* 2003). To address the functional consequences of the ERE sequence-dependent alterations on ERα- or ERβ-coactivator interaction, we have performed transient transfection experiments and quantitated the impact of representative coactivators on E_2-induced reporter gene expression from single copies of the vit A2, pS2, PR, and c-fos EREs. We report that different ERE sequences alter coactivator enhancement of E_2-induced transcriptional activity by ERα and ERβ. Further, differences in coactivator interaction between ERα and ERβ were also apparent. These data demonstrated that alterations in ERE sequence functionally impact coregulatory protein interaction with ERα and ERβ and that ERα and ERβ show subtype-dependent coactivator interactions.

Materials and methods

ER preparation and ER-ERE K_d determinations

Human ERα and ERβ1 were expressed from baculoviruses in Sf21 cells as described (Kulakosky *et al.* 2002). EMSAs were used to determine the affinity of E_2-occupied ERα and ERβ for the following EREs: EREc13: 5′-CCGGTCAGAG TGACCAG-3′; EREc38 (which is identical to the commonly used *Xenopus* vit A2 ERE (Peale *et al.* 1989)): 5′-CCAGGTCAGAGTGACCTGAGCTA AAATAACACATT-3′; PR1148: 5′-AGCCCTCC CTCCTGCGAGGTCACCA**GC**TC**T**TGGTGC CTGTTT-3′; pS2: 5′-CTTCCCCCTGCAAGG TCAGCGTG**G**CC**A**CCCCGTGAGCCACT-3′; and Fos-1211: 5′-AGCTTGGGCTGAG**C**C**G**G CAGCGTGACCCCGCATG-3′.

The underlined nucleotides correspond to the minimal core consensus ERE. The nucleotides in bold indicate an alteration in the consensus ERE. EREc38, PR1148, pS2, and Fos-1211 were cloned into the pGL3-promoter luciferase reporter vector (Promega, Madison, WI, USA) as previously described (Klinge *et al.* 1997). Details of the EMSA experiments to determine ER-ERE binding affinity are provided in Kulakosky *et al.* (2002), Tyulmenkov *et al.* (2000), and Tyulmenkov & Klinge (2001*a*,*b*).

Cell culture, transient transfection, and reporter assays

Chinese Hamster Ovary cells (CHO-K1) were purchased from ATCC (Manasas, VA, USA) and maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco/Invitrogen, Grand Island, NY, USA) supplemented with heat-treated, charcoal-stripped 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA). All other cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). For transient transfection, CHO-K1 cells were plated into 24-well plates at 1×10^5 cells/well with IMDM (without phenol red) supplemented with 10% charcoal-stripped fetal bovine serum. The cells were transfected when 80% confluent with 0·25 μg reporter construct containing the ERE, 5 ng pRL-CMV (RL=*Renilla* luciferase from Promega, Madison, WI, USA), 10 ng pCMV-human ERα or pCMV-rat ERβ, graciously provided by Drs B S Katzenellenbogen and J-A Gustafsson respectively. The expression plasmid for NCoR was provided by Dr M Bagchi (Zhang *et al.* 1998). The expression plasmid for SMRT was provided by Dr B G Rowan (Coleman *et al.* 2003). The expression plasmids for SRC-1 and SRA were provided by Dr B W O'Malley (Lanz *et al.* 1999). The expression plasmid for CBP was provided by Dr M Hadzopoulou-Cladaras (Dell & Hadzopoulou-Cladaras 1999). The expression plasmids for GRIP1, ACTR, CARM1, and PRMT1 were provided by Dr M Stallcup (Koh *et al.* 2001). The amounts of each of the coregulator plasmids used in transfection are indicated in the Figures and their legends. In experiments comparing the activity of a single coactivator with that achieved by co-transfecting two coactivators,

coactivators were run alone or in combination within the same transfection to obviate differences between transient transfections.

The transient transfections were performed using Transfast (Promega) as previously described (Klinge *et al.* 1999, 2000, Tyulmenkov *et al.* 2000). The total amount of DNA transfected was kept constant using pcDNA3 (Invitrogen, Carlsbad, CA, USA) as 'filler DNA'. Cells were treated, in triplicate, 24 h later with ethanol (EtOH, vehicle), 10 nM E_2 (Sigma, St Louis, MO, USA), 100 nM 4-hydroxytamoxifen (4-OHT) (Research Biochemicals International, Natick, MA, USA), or both E_2 and 4-OHT. Cells were harvested 30 h later and luciferase and *Renilla*-luciferase (RL-luc) activities assayed using the Promega dual luciferase reporter assay (Klinge *et al.* 1997, 1999, Tyulmenkov *et al.* 2000). All data for transient transfections were normalized by RL-luc to account for transfection efficiency.

Statistical analyses

Statistical analyses were performed on fold-induction data values from multiple experiments (minimally three) in which each treatment was run in triplicate using either two-tailed Student's *t*-test or one-way ANOVA followed by Dunn's multiple comparison or Dunnett's post-hoc test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

CHO-K1 cells were transfected with expression vectors for coactivators, corepressors, ERα, ERβ, and reporter vectors as described above. Whole cell extracts were prepared as described previously (Klinge *et al.*, 2000). Protein concentrations were determined by BioRad's DCC (Lowry) assay (BioRad, Hercules, CA, USA) using BSA as a standard. Equal amounts of protein (75 μg/lane) were separated by electrophoresis on 10% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Pall Corporation, Ann Arbor, MI, USA). The blots were probed with primary antibodies: SRC-1 (GeneTex, San Antonio, TX, USA); GRIP1 and ACTR (both from US Biological, Swampscott, MA, USA); β-actin (Amersham, Arlington Heights, IL, USA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostics, Inc., Flanders, NJ, USA). Immunoreactive complexes were visualized using SuperSignal West Pico Chemiluminescent substrate from Pierce (Rockford, IL, USA) on Eastman Kodak (Rochester, NY, USA) BioMax ML film. Data were quantitated from scanned films using Un-Scan-It software (Klinge *et al.* 2001).

GST-RID fusion protein preparation

The plasmids pGEX-2-TK-SRC-1 (219–399), pGEX-2TK-TIF2 (623–986), and pGEX-2TK-AIB1 (522–82) were graciously provided by Drs M Muylan and R Hilf of the University of Rochester (Yi *et al.* 2002). The plasmid pGEX-4T-1-NCoR was graciously provided by Dr A Hollenberg of Harvard University (Cohen *et al.* 2000). BL21 *E. coli* were used for expression of the GST-RID fusion proteins which were purified by GSH-Sepharose (Amersham Biosciences, Piscataway, NJ, USA) affinity chromatography as described previously (Klinge *et al.* 1997).

EMSA

Protein-DNA binding was measured by EMSA as previously reported (Klinge *et al.* 2001). Identical molar amounts of baculovirus-expressed human ERα or ERβ1, based on hydroxyapatite (HAP) assay results, were incubated with 150–500 fmol ^{32}P-labeled 50 bp oligomers EREc38, EREc13, pS2, PR1148, and Fos-1211. Binding reactions included 40 mM Tris–HCl (pH 7·5), 10% glycerol, 0·75 μg/μl BSA, and 0·02 μg/μl poly d(I-C) (Midland Certified Reagents, Midland, TX, USA), 111 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0·5 mM phenylmethylsulfonyl fluoride. ERα- and ERβ-specific antibodies (G-20 and Y19 from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were included in selected reactions. Dried EMSA gels were analyzed using a Packard Instruments InstantImager (Meridian, CT, USA) and associated software, Packard Imager for Windows v2·04 (Tyulmenkov & Klinge 2001*b*).

Results

ERE sequence impacts ERα- and ERβ-mediated transcriptional activity

Transient cotransfection assays were performed in CHO-K1 cells to determine how the nucleotide sequence of the ERE affects the enhancer activity of ERα and ERβ. This cell line was selected

because it requires exogenous ERα or ERβ to activate ERE-driven reporter gene expression (McInerney *et al.* 1996) and thus allows evaluation of the transcriptional response of each ER subtype in isolation with each ERE. We selected five different ERE-driven luciferase reporter constructs to examine possible differences in transactivation potential between ERα and ERβ based on DNA sequence (see Materials and methods for sequences). EREc38 is a consensus ERE that includes the commonly used *Xenopus* vit A2 ERE palindrome (Klinge *et al.* 1992). EREc13 is the minimal consensus 13 bp palindromic ERE (Klein-Hitpass *et al.* 1988). The natural, non-consensus, imperfectly palindromic EREs were taken from the human pS2, *c-fos*, and PR genes (Kulakosky *et al.* 2002). The cells were treated with EtOH vehicle control, 10 nM E_2, 100 nM 4-OHT, or both, and luciferase and RL-luc activities were assayed (Fig. 1). For each of the four EREs tested, E_2 increased luciferase expression above the basal level for both ERα and ERβ. Luciferase activity detected in the presence of 4-OHT was either at or below basal expression. Co-treatment with E_2 and 4-OHT inhibited E_2-induced reporter activity, indicating that the E_2-induced activation is mediated specifically by ERα and ERβ. ERα showed a greater E_2-induced fold-induction of reporter gene activity with the EREc38, EREc13, and pS2 EREs compared with ERβ. However, the fold-induction of luciferase by ERα and ERβ was similar on the Fos-1211 and PR1148 EREs. These results indicated that the ERE sequence differentially impacts the E_2-dependent transcriptional activities of ERα and ERβ.

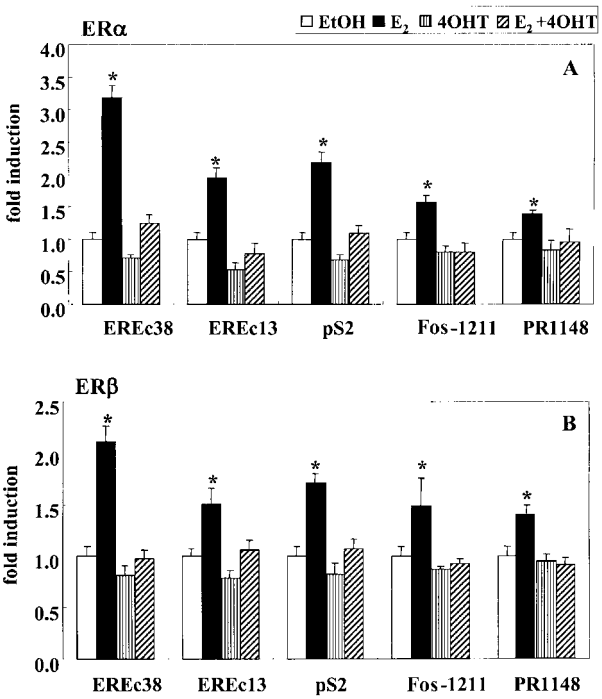
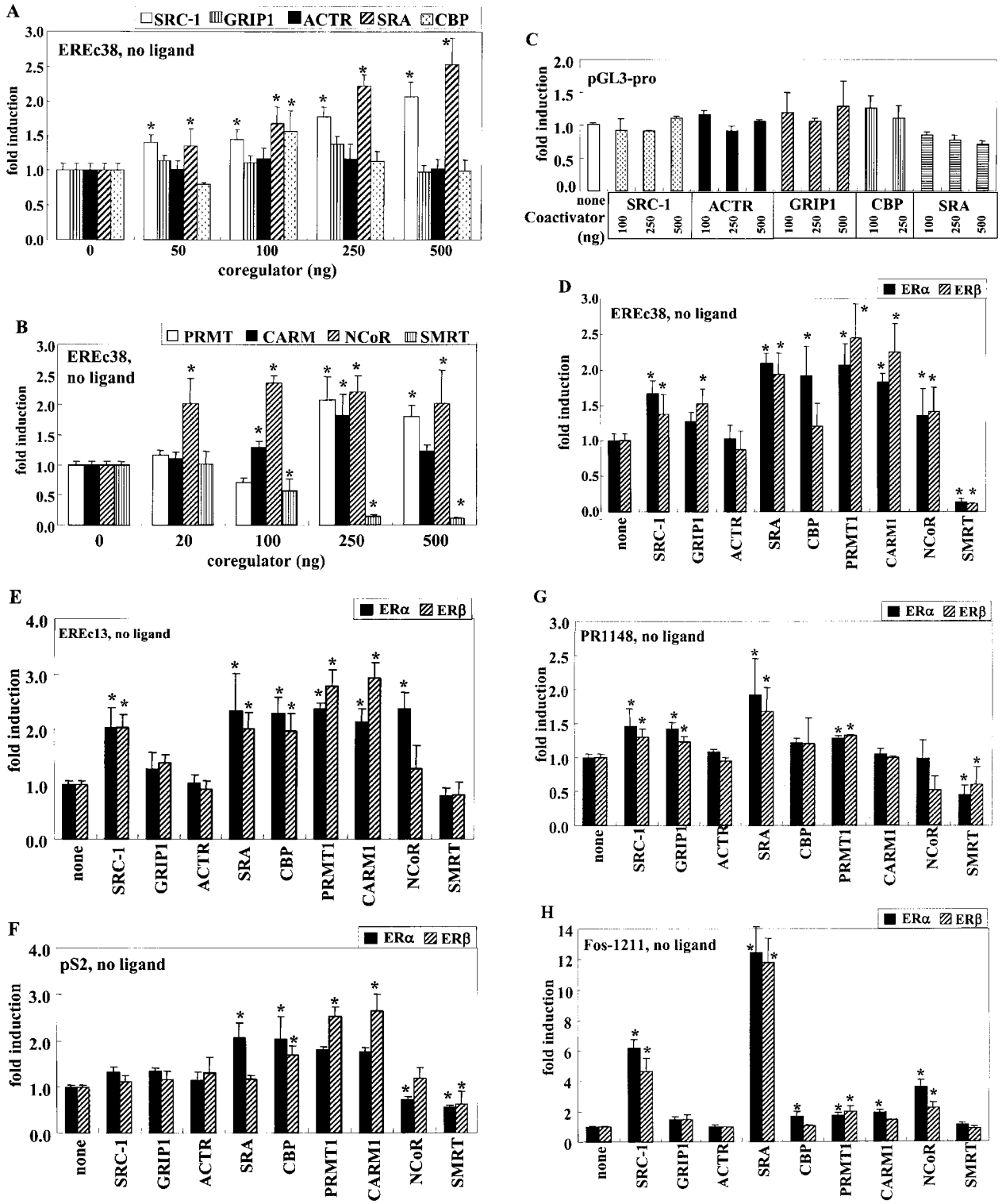


Figure 1 ERE sequence impacts ERα and ERβ transcriptional activity. CHO-K1 cells were co-transfected with (A) ERα or (B) ERβ plus the indicated pGL3-ERE-luciferase reporter and pRL-CMV as described in Materials and methods. Twenty-four hours after transfection, the cells were treated with EtOH, 10 nM E_2, 100 nM 4-OHT, or 10 nM E_2 plus 100 nM 4-OHT for 30 h. Cell extracts were prepared and assayed as described in Materials and methods. Data are displayed as luciferase activity divided by the RL-luc activity in each well and normalized by EtOH control (which is set to 1). Within each experiment, each treatment was performed in triplicate. The data shown are the means±S.E.M. from at least 11 separate experiments. *$P<0{\cdot}05$, E_2 values that are statistically different from the EtOH control value.

Coactivators impact basal transcription activity

We have previously reported that the ERE nucleotide sequence altered ERα conformation as assessed by protease digestion studies (Klinge *et al.* 2001, Tyulmenkov & Klinge 2001*a*). Here we tested the hypothesis that the observed ERE sequence-induced alterations in ER conformation may alter ER-coregulator interactions *in vivo*. This is the first study to directly compare coregulator interactions with ERα and ERβ in response to single copy EREs in the context of their natural flanking sequence in a functional assay. Further, although coactivators have been shown to impact ERα and ERβ activity in transfected cell lines using different ERE reporters, most prominently multiple tandem copies of the vit A2 ERE, few investigators have separated the effect of coregulators on the basal activity of ER on the reporter gene assayed versus that stimulated by E_2 (reviewed in Klinge 2003).

First, we examined the effect of coregulators on basal, ligand-independent ERα activity by measuring luciferase expression from each ERE (Fig. 2). CHO-K1 contains endogenous SRC-1, GRIP1, ACTR, CBP, and SMRT (Western data not shown). To account for endogenous expression of coregulators, each ER-ERE combination tested



was treated with ethanol in the absence of co-transfected coregulators. This control level of reporter activity was set to 1. SRC-1 and SRA increased basal ERα activity in a concentration-dependent manner from EREc38 (Fig. 2A). CBP significantly stimulated basal activity only at 100 ng. PRMT1, CARM1, and NCoR stimulated basal ERα transcriptional activity from EREc38 whereas SMRT repressed the basal ERα activity in a concentration-dependent manner (Fig. 2B). Noteworthy is the fact that none of the coregulators affected the basal activity of ERα or ERβ on the parental pGL3-pro-luciferase vector without EREs (Fig. 2C and data not shown). None of the coregulators with the exception of CBP had any effect on either firefly luciferase activity from pGL3–1 EREc38 or the other reporter vectors or RL-luc activity. Since CBP did not stimulate luciferase activity from the pGL3-pro plasmid (Fig. 2C), despite the presence of one cyclic AMP response element binding protein (CREB) binding site in the luciferase gene and another in the ampicillain resistance gene (Promega; personal communication), the stimulation of luciferase activity from pGL3–1 EREc38 by CBP must be mediated by the inclusion of EREc38 which contains a cyclic AMP response element (CRE). Nonetheless, because our data are normalized for CBP stimulation on the ERE reporters in the absence of ER, the stimulation of ligand-independent ERα basal activity by SRC-1, SRA, CBP, PRMT1, CARM1, and NCoR (Fig. 2A and B) is mediated by ERE-ERα interaction. Based on the work of other investigators (Webb *et al.* 2003), we suggest that these effects of coactivators on unliganded ER may be mediated through the N-terminal activation domain (AF-1) which is ligand independent.

Next, we compared the ligand-independent activity of ERα and ERβ with a fixed amount of each coregulator on EREc38 in transfected CHO-K1 cells (Fig. 2D). SRC-1, SRA, PRMT1, CARM1, and NCoR stimulated the basal activity of ERα and ERβ. GRIP1 stimulated unliganded ERβ but not ERα and conversely CBP stimulated the activity of unliganded ERα but not ERβ. SMRT repressed the basal activity of both unliganded ERα and ERβ.

To address the role of the ERE nucleotide sequence in the effect of coregulators on ligand-independent ERα and ERβ, cells were transfected with EREc13, pS2, PR1148, and Fos-1211 EREs and a fixed amount of each coregulator (Fig. 2E–H). Each ERE showed both ER subtype- and coactivator-specific differences in transcriptional response. The most striking ligand-independent stimulation of ERα and ERβ activity was with SRA and SRC-1 on Fos-1211. ACTR did not stimulate either ERα or ERβ on any of the EREs. ERα and ERβ showed different responses to NCoR on EREc13 and pS2 and to PRMT1 and CARM1 on pS2.

To address the role of E_2 in mediating functional interaction of ERα and ERβ with coregulators, in all subsequent experiments cells were transfected with each coregulator and treated with EtOH and 10 nM E_2; the luciferase activity detected with E_2 was normalized by that for EtOH. Thus, any effect of a coregulator on basal, ligand-independent ER transcription was excluded from subsequent analysis. This allows comparison of the effect of a coregulator on ligand-activated ER transcription independent of effect of that coregulator on ligand-independent (basal) transcription from the reporter.

ERE sequence impacts coactivator-mediated transactivation by E_2-ERα and E_2-ERβ

Next, we compared how different ERE sequences affected the ability of coregulators to affect E_2-stimulated activity with ERα or ERβ. Figure 3A shows that of the tested coactivators, only SRC-1 stimulated E_2-induced ERα activity on the vit A2

Figure 2 Coregulators influence ligand-independent ER activity. In (A and B) CHO-K1 cells were transfected with ERα, EREc38-luciferase reporter, and the amounts of the indicated coactivators. In (C) CHO-K1 cells were transfected with ERα, pGL3-pro-luciferase reporter (no EREs), and the amounts of the indicated coactivators. In (D–H) CHO-K1 cells were transfected with the indicated ERE-luciferase reporter, ERα or ERβ, and 250 ng of the indicated coregulators. All cells were co-transfected with RL-luc reporter control. Cells were treated with EtOH for 30 h and processed as described in Fig. 1 and in Materials and methods. Values are the fold-induction over EtOH activity in the absence of added coregulator and are the means±S.E.M. of three to eight different experiments. *$P < 0{\cdot}05$, values that are statistically different from the control value.

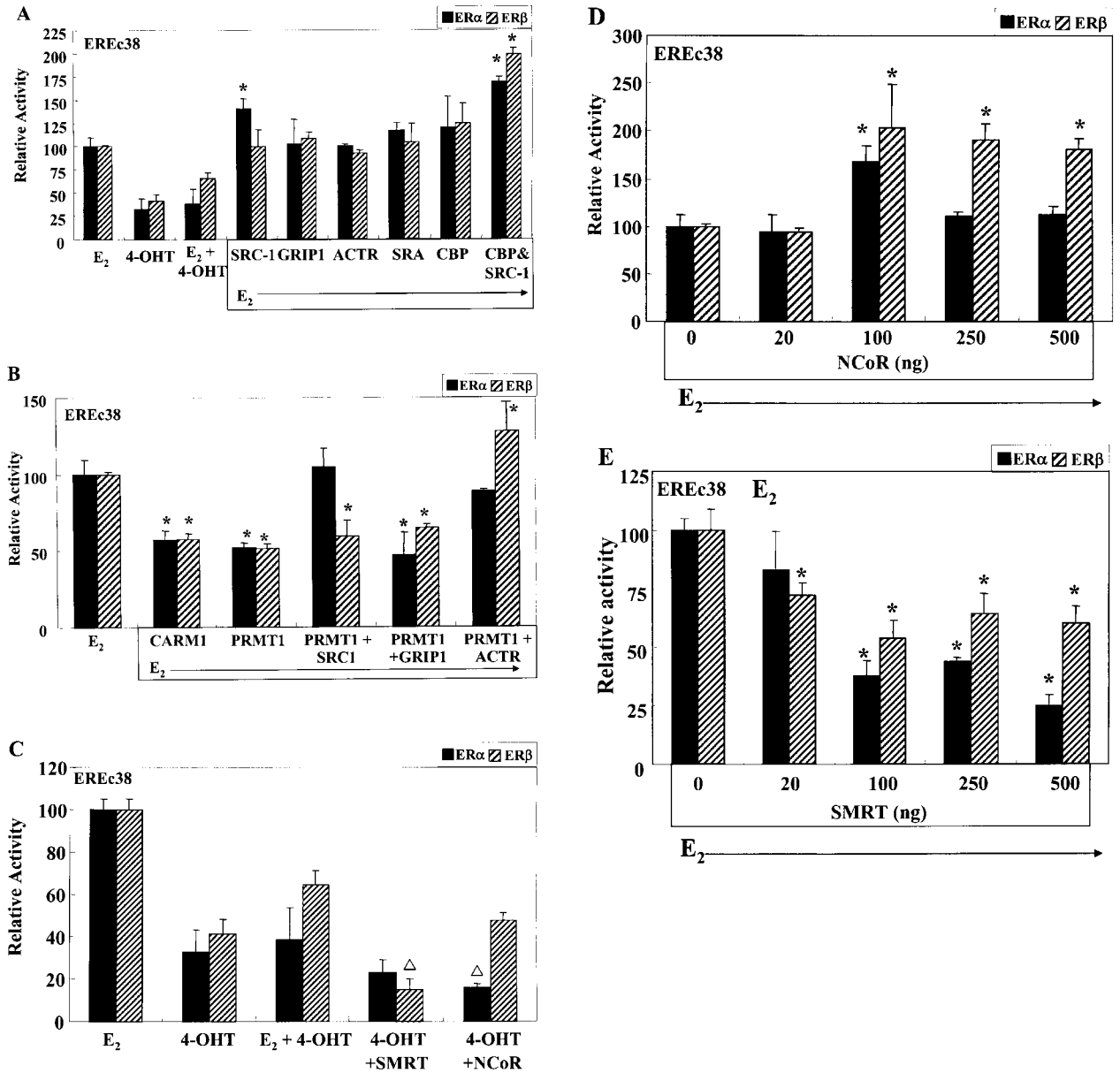


Figure 3 Transcriptional response of ERα and ERβ to coregulators on EREc38. CHO-K1 cells were transfected with ERα or ERβ and EREc38-luciferase reporter, pRL-CMV, and 250 ng of the indicated coregulator as described in Materials and methods. Cells were treated with EtOH, 10 nM E_2, 100 nM 4-OHT, or both E_2 and 4-OHT, as indicated, for 30 h and processed as described in Fig. 1 and in Materials and methods. The maximal activity with 10 nM E_2 was set at 100 (absolute fold values are shown in Fig. 1). Values are the means±S.E.M. of three to eight different experiments. (A, B, D, and E) $*P<0{\cdot}05$, values that are statistically different from the E_2-induced value for that receptor subtype in the absence of coregulator. (C) Δ $P<0{\cdot}05$, values that are significantly different (Student's *t*-test) between the 4-OHT activity without and with SMRT or NCoR.

ERE (EREc38). Singly, none of the coactivators stimulated ERβ activity. The combination of CBP and SRC-1 synergistically activated E_2-induced ERβ but not ERα activity. In contrast, both CARM1 and PRMT1 inhibited ERα- and ERβ-induced transcriptional activity (Fig. 3B). This result contrasts to the stimulation of basal activity of ERα and ERβ by CARM1 and PRMT1.

Cotransfection of SRC-1 relieved PRMT1-mediated repression of ERα, but not ERβ activity. This result agrees with the ERα-selective stimulation by SRC-1 shown in Fig. 3A. ACTR relieved PRMT1-mediated repression of both ERα and ERβ. GRIP1 did not relieve the PRMT1-mediated repression of either ERα and ERβ.

4-OHT binds ERα and ERβ with comparable affinity (Kuiper *et al.* 1997). However, 4-OHT only exhibits agonist activity with ERα at EREs in certain cell types (Weatherman & Scanlan 2001). Our experiments show that 4-OHT had no agonist activity with either ERα or ERβ and inhibited E_2-induced transcription by both ERα and ERβ in CHO-K1 cells (Fig. 3C). The corepressor SMRT decreased 4-OHT activity with ERβ. In contrast, the corepressor NCoR further decreased 4-OHT-ERα but not ERβ activity. These results indicate that 4-OHT-occupied ERα and ERβ differ in their interactions with corepressors SMRT and NCoR. Our data are in agreement with phage display experiments showing differences in the conformation of 4-OHT-occupied ERα and ERβ *in vitro* (Paige *et al.* 1999).

Recently, the corepressors NCoR and SMRT were shown to interact directly with ACTR and facilitate thyroid receptor-ACTR binding *in vitro* and in a mammalian two-hybrid assay (Li *et al.* 2002). We tested the hypothesis that addition of SMRT or NCoR to cells transfected with ERα or ERβ and treated with E_2 would result in increased reporter gene expression. NCoR increased E_2-induced activity by ERα only with the addition of 100 ng. In contrast, ERβ activity was increased with 100 ng and higher amounts of NCoR (Fig. 3D). SMRT decreased E_2-induced activity by ERα or ERβ in a concentration-dependent manner that appeared to saturate for ERβ at 100 ng (Fig. 3E). These data indicated that under the assay conditions used NCoR acts as a coactivator whereas SMRT acts as a corepressor of ERα and ERβ.

EREc13, the minimal consensus ERE (Klein-Hitpass *et al.* 1988), binds ERα and ERβ with significantly lower affinity than ERE palindromes containing 15 bp or more, i.e. K_d 1·1 nM and 1·7 nM versus 0·11 nM and 0·13 nM for ERα and ERβ respectively (Table 1 and (Kulakosky *et al.* 2002)). GRIP1, ACTR, and SRA increased E_2-ERα activity on EREc13 (Fig. 4). In contrast, none of the coactivators stimulated E_2-ERβ activity. These results differ from stimulation of unliganded ERβ by SRA (Fig. 2E). However, the combination of SRC-1 and CBP stimulated E_2-induced ERβ activity. NCoR stimulated E_2-induced ERβ but not ERα activity. These data indicated that ERα and ERβ show functional differences in coactivator interaction on a minimal ERE reporter in E_2-treated cells, a result concordant with differential interaction of small peptides mimicking coregulator interaction sequences with ERα and ERβ *in vitro* (Hall *et al.* 2000). Furthermore, comparison of the coactivator stimulation of ERα and ERβ on EREc38 versus EREc13 (Table 1) indicates that the length of the ERE palindrome differentially impacts ERα and ERβ interaction with coregulators, a result predicated by work using phage display to examine conformational differences between ERα and ERβ bound to different EREs (Hall *et al.* 2002).

Finally, we examined the effect of coregulators on the activities of ERα and ERβ bound to natural, non-palindromic EREs from the human pS2, PR, and *c-fos* genes (Fig. 5). Notably, certain coactivators stimulated E_2-ER transcription in an ER subtype- and ERE sequence-dependent manner. E_2-induced ERα activity was enhanced 30% by ACTR on pS2, but not on Fos-1211 or PR1148. E_2-induced ERβ activity on pS2 was enhanced by SRC-1, GRIP1, SRA, CBP, CARM1, and PRMT1 in the presence of GRIP1. In contrast, only CBP stimulated E_2-induced ERβ activity on PR1148. E_2-induced ERα activity on PR1148 was inhibited by cotransfection with SRA. ERβ activity on PR1148 was reduced by CARM1 and PRMT1. PRMT1 was reported to have stronger coactivator activity when co-transfected with p160 coactivators compared with CARM1 (Koh *et al.* 2001). The inhibitory effect of PRMT1 on E_2-induced ERβ activity on PR1148 was relieved by cotransfection with ACTR, but not by SRC-1 or GRIP1.

Coactivators showed the least impact on E_2-stimulated ERα or ERβ activity on Fos-1211 (Fig. 5C). This is in contrast to the high stimulation of unliganded ERα or ERβ activity on Fos-1211 (Fig. 2H). Only GRIP1 and SRA enhanced E_2-induced ERα and ERβ activity respectively (Fig. 5C). SRC-1 inhibited E_2-ERα activity. CBP inhibited E_2-induced ERα and ERβ activity. The lack of effect of coactivators on E_2-induced reporter activity from the non-palindromic Fos-1211 ERE may be the result of the reduced ER binding affinity (Table 1) which would theoretically

Table 1 Effect of ERE sequence on E_2-induced transcriptional activity by ERα and ERβ. K_d values are the means±S.E.M. of four to six separate EMSA determinations as described (Tyulmenkov *et al.* 2000, Klinge *et al.* 2001, Tyulmenkov & Klinge 2001b, Kulakosky *et al.* 2002). The effect of the indicated coactivator on E_2-stimulated ERα or ERβ transcriptional activity is a summary of the relevant data from the transient transfection assays performed in CHO-K1 cells as shown in Figs 3–6

	ERE palindrome (c=consensus, perfectly palindromic ERE (Klinge 2001))	**ERα K_d** (nM)	**ERβ K_d** (nM)	**ERα stimulated by**	**ERα inhibited by**	**ERβ stimulated by**	**ERβ inhibited by**
ERE							
EREc38	EREc19	0·11±0·02	0·64±0·02	SRC-1 NCoR	CARM1 PRMT1 (PRMT+ GRIP1)	NCoR SMRT (PRMT1+ ACTR)	CARM1 PRMT1 (PRMT+ SRC-1) (PRMT+ GRIP1)
EREc13	EREc13	10·7±0·07	15·5±2·1	SRA, GRIP1		NCoR (CBP+ SRC-1)	
pS2	EREc15, two nucleotide changes in the 3′ half-site	1·06±0·02	3·0±0·6	ACTR	NCoR	SRC-1 GRIP1 SRA CBP CARM1 (PRMT1+ GRIP1)	(PRMT1+ ACTR)
PR1148	EREc15, four nucleotide changes in the 3′ half-site	3·3±0·3	29·1±5·4	SRC-1 CBP	SRA SMRT	CBP NCoR	CARM1 PRMT1 (PRMT1+ SRC-1) (PRMT1+ GRIP1) SMRT
Fos-1211	EREc13, two nucleotide changes in the 5′ half-site	328±38	240±24	GRIP1	SRC-1 CBP NCoR	SRA	CBP NCoR SMRT

decrease ER-ERE occupancy ('on time') and therefore reduce assembly of the coactivator complex.

We also tested the effect of addition of NCoR or SMRT on E_2-induced reporter activity from the non-palindromic EREs (Fig. 5D). As with the data on coactivators, NCoR and SMRT differed in their effect on transcription in an ER subtype- and ERE sequence-dependent manner. NCoR decreased E_2-ERα activity from pS2 and Fos-1211, but not PR1148. NCoR also decreased E_2-ERβ activity from Fos-1211. NCoR enhanced E_2-ERβ activity with PR1148. SMRT inhibited E_2-induced ERα activity from PR1148 and ERβ activity from Fos-1211 and PR1148. We concluded that SMRT and NCoR differentially impact E_2-induced transcriptional activity in an ER subtype- and ERE sequence-dependent manner.

A possible explanation for the differences detected between the effects of the coactivators on ERα or ERβ transcriptional activity is altered expression of the coactivators under different experimental conditions. To determine whether the expression of the coregulators was equal in transfected cells, Western blots were performed (Fig. 6). Densitometric analysis of the SRC-1, GRIP1, or ACTR/β-actin ratio in repeated transient transfection experiments revealed no statistical differences in the amounts of coactivator expressed in cells transfected with either ERα or ERβ and that there was no effect of ERE sequence on the expression of transfected SRC-1, GRIP1, or

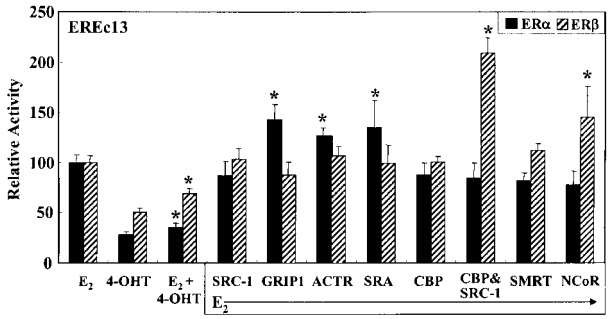


Figure 4 Transcriptional response of ERα and ERβ to coregulators on EREc13. CHO-K1 cells were transfected with ERα or ERβ, EREc13-luciferase, pRL-CMV, and 250 ng of the indicated coregulators as described in Materials and methods and Figs 1 and 3. Values are the means±S.E.M. of three to nine different experiments. *$P < 0.05$, values that are statistically different from the E_2-induced value for that receptor subtype in the absence of coregulator.

ACTR in CHO-K1 cells. This obviates differences in coregulator expression as an explanation for the observed differences in transcriptional activity seen with the different ERE reporters and between ERα or ERβ.

A possible explanation for the small differences in the effects of the coactivators on ERα or ERβ transcriptional activity with different EREs is that the if endogenous levels of a particular coregulator are already high, our experiments may not pick up any further effect of transfection of additional coregulator. Evidence that the effect of ER-coregulator interactions is not already saturated can be seen in Fig. 2 for SRC-1, SRA, CBP, CARM1, PRMT1, NCoR, and SMRT which stimulated unliganded ERα and in some cases ERβ transcriptional activity. Further, GRIP1 stimulated unliganded ERα and ERβ on PR1148 (Fig. 2 G) and ACTR stimulated ERα activity on EREc13 (Fig. 4) and pS2 (Fig. 5A). Thus, each of the coregulators studied stimulated ERα and ERβ activity on at least one transfected ERE reporter. If the response was already saturated for a particular coregulator, then no added stimulation would be seen under any circumstance.

Synergism of combinations of coactivators with ERα and ERβ varies with ERE sequence

Coactivators including CBP, SRA, CARM1, and PRMT1 are recruited to target gene promoters through their interaction with p160 family coactivators interacting directly with ER and other nuclear receptors (NR) (Teyssier *et al.* 2002). To test whether SRA synergized with p160 coactivators to stimulate E_2-dependent transcription in an ERE sequence-dependent manner, CHO-K1 cells were transfected with either ERα or ERβ, plus SRA, alone or in combination with SRC-1, GRIP1, or ACTR with different ERE reporters (Fig. 7). SRA showed no synergism with ACTR (compare with SRA and ACTR alone in Figs 3A, 4, and 5). GRIP1 synergized with SRA for ERα only on the Fos-1211 ERE (compare with GRIP1 and SRA alone in Fig. 5C). In contrast, SRA synergized with GRIP1 for ERβ on EREc38, EREc13, and Fos-1211 (compare with data in Figs 3A, 4, and 5C). Lastly, SRA synergized with SRC-1 for ERβ on EREc13 (compare with SRA and SRC-1 alone in Fig. 4).

Coactivator RIDs show ER subtype- and ERE sequence-dependent ER interaction *in vitro*

Since coregulators showed differential effects on ERα and ERβ transcriptional activity in an ERE sequence-dependent manner in transfected cells, we tested whether the RIDs of selected p160 coactivators showed ERE sequence-dependent interaction with ERα and ERβ in EMSAs. GST-fusion proteins containing the RIDs of SRC-1, GRIP1, ACTR, or NCoR were incubated with E_2-occupied ERα or ERβ and different EREs *in vitro*. The sequence of the GST-RIDs and their location within the coregulator is shown in Table 2. As a negative control, each GST-RID was incubated with each ERE in the absence of ER. None of the GST-RIDs interacted with the EREs (Fig. 8 and data not shown). Figure 8 shows representative EMSAs for ERα and ERβ binding to the pS2 ERE with or without added GST-ACTR or GST-GRIP1.

Addition of GST-ACTR resulted in the appearance of an ERE-bound complex with slowed migration for both ERα and ERβ bound to pS2-ERE (Fig. 8) and EREc38 (Fig. 9A and B). In contrast, only the migration of ERα-EREc13 was slowed by GST-ACTR (Fig. 9C and D). The amount of 'supershifted', i.e. complex with slowed migration, ACTR-ERα-pS2 complex was greater than for ERβ (Fig. 8C and D). The total amount of ERα-bound EREc13 pS2, and EREc38 complexes

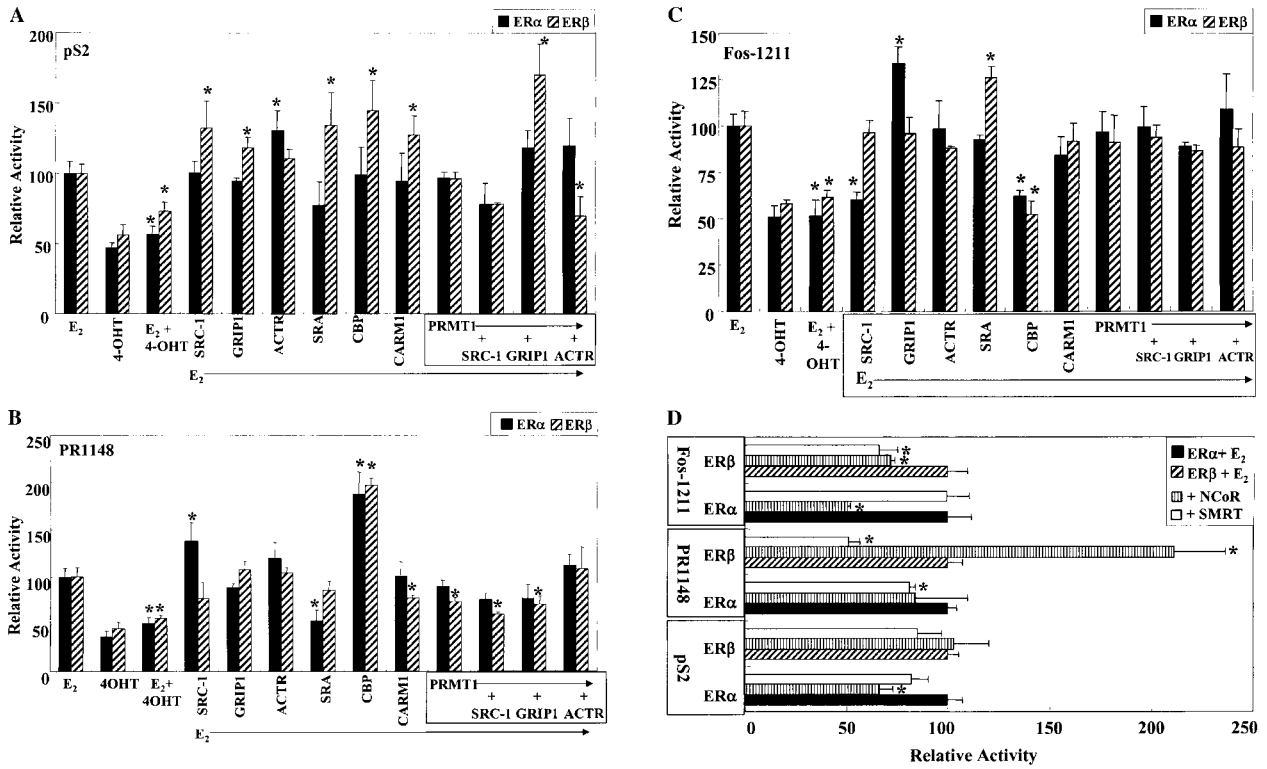


Figure 5 Natural variations on ERE palindrome sequence impact the effect of coactivators and corepressors on E_2-induced ERα and ERβ transcription. CHO-K1 cells were transfected with ERα or ERβ and (A) pS2, (B) PR1148, or (C) Fos-1211 ERE-luciferase reporters plus pRL-CMV and 250 ng of each of the indicated coregulators as described in Materials and methods. (D) The impact of the addition of either NCoR or SMRT on E_2-induced ERα or ERβ activity from the indicated ERE-luciferase reporter. Cells were treated with 10 nM E_2, 100 nM 4-OHT, or both (as indicated) for 30 h and processed as described in Fig. 1 and in Materials and methods. The maximal activity with 10 nM E_2 was set at 100% and the actual fold-induction values are shown in Fig. 1A and 1B for E_2-ERα and ERβ respectively. Values are the means±S.E.M. of three to nine different experiments. $^*P<0{\cdot}05$, values that are statistically different from the E_2-induced value for that receptor subtype in the absence of coregulator.

was increased with the addition of GST-ACTR. For ERβ, GST-ACTR increased the total amount of ERβ-bound pS2 complex, but not ERβ-bound EREc38 or EREc13 complexes. This indicated that GST-ACTR shows preferential interaction with ERα rather than ERβ bound to EREc13 and EREc38. Despite a previous report that the GST-SRC-1 and -GRIP1 constructs used here interacted stably with ERα and ERβ in EMSA (Yi *et al.* 2002), neither formed a complex with ERα or ERβ for any of the tested EREs and had no significant effect on ER-ERE binding (Figs 8C and 9). We concluded that the GRIP1 and SRC-1 RIDs used in the EMSAs do not stably bind ER under our experimental conditions. None of the GST-RIDs supershifted the ERα- or ERβ-Fos-1211 or PR1148 complexes (Table 3 and data not shown). Lastly, GST-NCoR RID showed no visible effect on the migration of the ERβ-EREc38 complex (data not shown) and no effect on ERβ-EREc13 binding (Fig. 9D).

Discussion

Lefstin & Yamamoto (1998) proposed that DNA elements recognized by nuclear transcription factors contain information that is interpreted by the bound regulator. Based on their model, we postulated that DNA acts as an allosteric modulator that, when bound by ER, alters ER conformation, resulting in altered ERE binding affinity and transcriptional activity (Klinge 1999). In turn, ERE-induced changes in ER conformation were

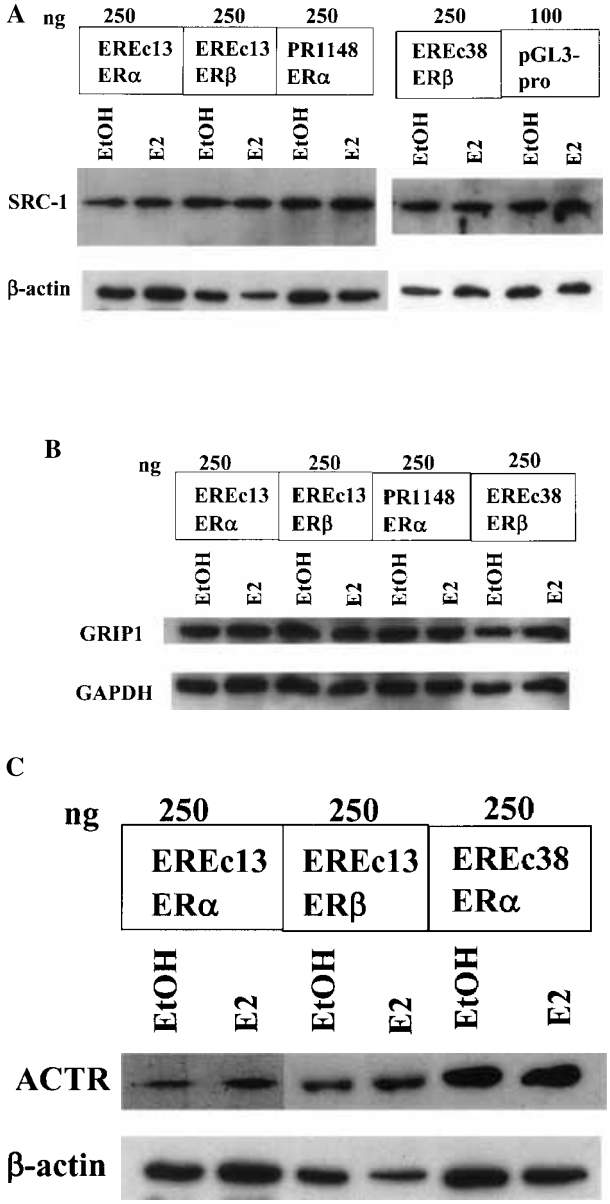


Figure 6 Coactivator expression in transiently transfected CHO-K1 cells. CHO-K1 cells were transfected with the indicated amount of the expression vector, ER subtype, and ERE. Western blots were performed on cell extracts using antibodies against (A) SRC-1 and β-actin, (B) GRIP1 and GAPDH, and (C) ACTR and β-actin. These blots are representative of experiments that were repeated twice.

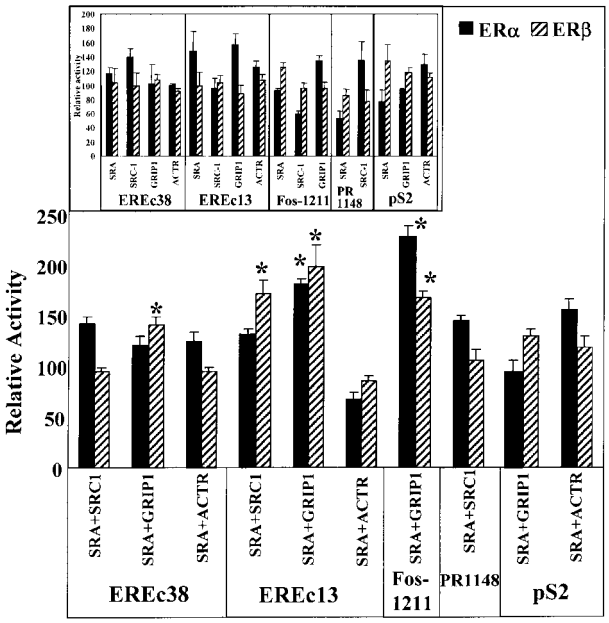


Figure 7 Synergism between SRA and p160 coregulators depends on ER subtype and ERE sequence. CHO-K1 cells were transfected with ERα or ERβ and the indicated ERE-luciferase reporters plus pRL-CMV and 250 ng SRA plus each of the indicated p160 coactivators as described in Materials and methods and Fig. 1. The maximal activity with 10 nM E_2 was set at 100. Values are the means±S.E.M. of three different experiments. *$P<0{\cdot}05$, values that are statistically different from both the E_2-induced value for that receptor subtype/ERE combination with either SRA or the indicated coactivator alone. The inset shows the relative activity of each coactivator alone with E_2-ERα or E_2-ERβ on the indicate ERE. These data are taken from Figs 3, 4, and 5.

predicted to alter ER affinity for other 'ligands', such as coactivators or corepressors (Klinge *et al.* 2001). In fact, we detected conformational differences in ER depending on both ligand and the bound ERE sequence in protease digestion experiments (Klinge 1999, Bowers *et al.* 2000, Tyulmenkov & Klinge 2000*b*, 2001*a*, Tyulmenkov *et al.* 2000, Klinge *et al.* 2001, Ramsey & Klinge 2001). Other protease digestion as well as other *in vitro* data from the Nardulli (Wood *et al.* 1998, 2001, Loven *et al.* 2001*a*,*b*), McDonnell (Hall *et al.* 2002), and Shapiro (Krieg *et al.* 2004) laboratories have also demonstrated that the ERE sequence and ligand impact ERα and ERβ conformation *in vitro*. Here we present the first cell-based assay, as a mimic of *in vivo* conditions, to address how differences in ERE sequence impact the functional interaction of ERα and ERβ with coactivator and corepressor proteins.

To separate the effects of ERE sequence on ER-coactivator interaction from the interaction of that coactivator with other transcription factors in a

Table 2 GST-RID sequences utilized in the EMSA. These constructs were prepared and used in EMSAs as described in Materials and methods

	Amino acid location of RID	Amino acid sequence
Cointegrator		
SRC-1	219–399	EVMQCFTVSQPKSIQEDGEDFQSCLICIARRLPRPPAITGV ESFMTKQDTTGKIISIDTSSLRAAGRTGWEDLVRKCIYAFF QPQGREPSYARQLFQEVMTRGTASSPSYRFILNDGTMLSA HTKCKLCYPQSPDMQPFIMGIHIIDREHSGLSPQDDTNSG MSIPRVNPSVNPSISPAH
GRIP1	623–986	VSSERADGQSRLHDSKGQTK**LLQLL**TTKSDQMEPSPLAS SLSDTNKDSTGSLPGSGSTHGTSLKEKHKI**LHRLL**QDSSS PVDLAKLTAEATGKDLSQESSSTAPGSEVTIKQEPVSPKK KENAL**LRYLL**DKDDTKDIGLPEITPKLERLDSKTDPASNT KLIAMKTEKEEMSFEPGDQPGSELDNLEEILDDLQNSQLP QLFPDTRPGAPAGSVDKQAIINDLMQLTAENSPVTPVGA QKTALRISQSTFNNPRPGQLGRLLPNQNLPLDITLQSPTGA GPFPPIRNSSPYSVIPQPGMMGNQGMIGNQGNLGNSSTG MIGNSASRPTMPSGEWAPQSSAVRVTCAATTSAMNRPVQ GGMIRNPAA
ACTR	522–827	SLSALQAISEGVGTSLLSTLSSPGPKLDNSPNMNITQPSKV SNQDSKSPLGFYCDQNPVESSMCQSNSRDHLSDKESKESS VEGAENQRGPLESKGHKK**LLQLL**TCSSDDRGHSSLTNSP LDSSCKESSVSVTSPSGVSSSTSGGVSSTSNMHGSLLQEK HRI**LHKLL**QNGNSPAEVAKITAEATGKDTSSITSCGDGNV VKQEQLSPKKKENNAL**LRYLL**DRDDPSDALSKELQPQVE GVDNKMSQCTSSTIPSSSQEKDPKIKTETSEEGSGDLDNL DAILGDLTSSDFYNNSISSNGSHLGT
NCoR	2063–2300	FARNQVSSQTPQQPPTSTFQNSPSALVSTPVRTKTSNRYSP ESQAQSVHHQRPGSRVSPENLVDKSRGSRPGKSPERSHVS SEPYEPISPPQVPVVHEKQDSLLLLSQRGAEPAEQRNDAR SPGSISYLPSFFTKLENTSPMVKSKKQEIFRKLNSSGGGDS DMAAAQPGTEIFNLPAVTTSGSVSSRGHSFADPASNLG**LE DII**RKALMGSFDDKVEDHGVVMSQPMGVVPGTANTS

complex promoter, we used a simple model system in which each ERE, plus a relatively short stretch (6–20 bp) of nucleotides identical to those flanking that ERE in the natural human genes for pS2, c-Fos, and PR, was inserted in the same location in the multicloning region of a luciferase reporter gene. This obviates difficulties in data interpretation due to different distances between the ERE and transcription start site (Sathya *et al.* 1997, Nordeen *et al.* 1998) and focuses our study on the role of the ERE along with its natural flanking sequence in ER-coregulator interaction. Importantly, we evaluated ER-coregulator activity at a single copy of each ERE rather than multiple tandem copies of the vit A2 ERE. By using CHO-K1 cells that do not express ERα or ERβ (McInerney *et al.* 1996), we separated effects of coactivators and EREs on ERα versus ERβ. The importance of our study is that it is the first to examine ERβ-ERE interactions in a functional assay in direct comparison with ERα activity on the same single copy EREs in the context of their natural flanking sequence.

Our experiments have demonstrated that the sequence of the ERE impacts the functional interaction of ERα and ERβ with coactivators SRC-1, GRIP1, ACTR, CBP, and SRA, the coregulators CARM1 and PRMT1, and corepressors SMRT and NCoR in transiently transfected CHO-K1 cells. Notably, unliganded ER interacts with coregulators in an ERE sequence-dependent manner. Similar findings for CBP and SRC-1 were recently reported by others (Dutertre & Smith 2003). A brief summary model highlighting some of the ERE-specific differences between unliganded and E_2-occupied ER interaction with coregulators detected in our experiments is presented in Fig. 10. Although the affinity of ERα or ERβ binding to an

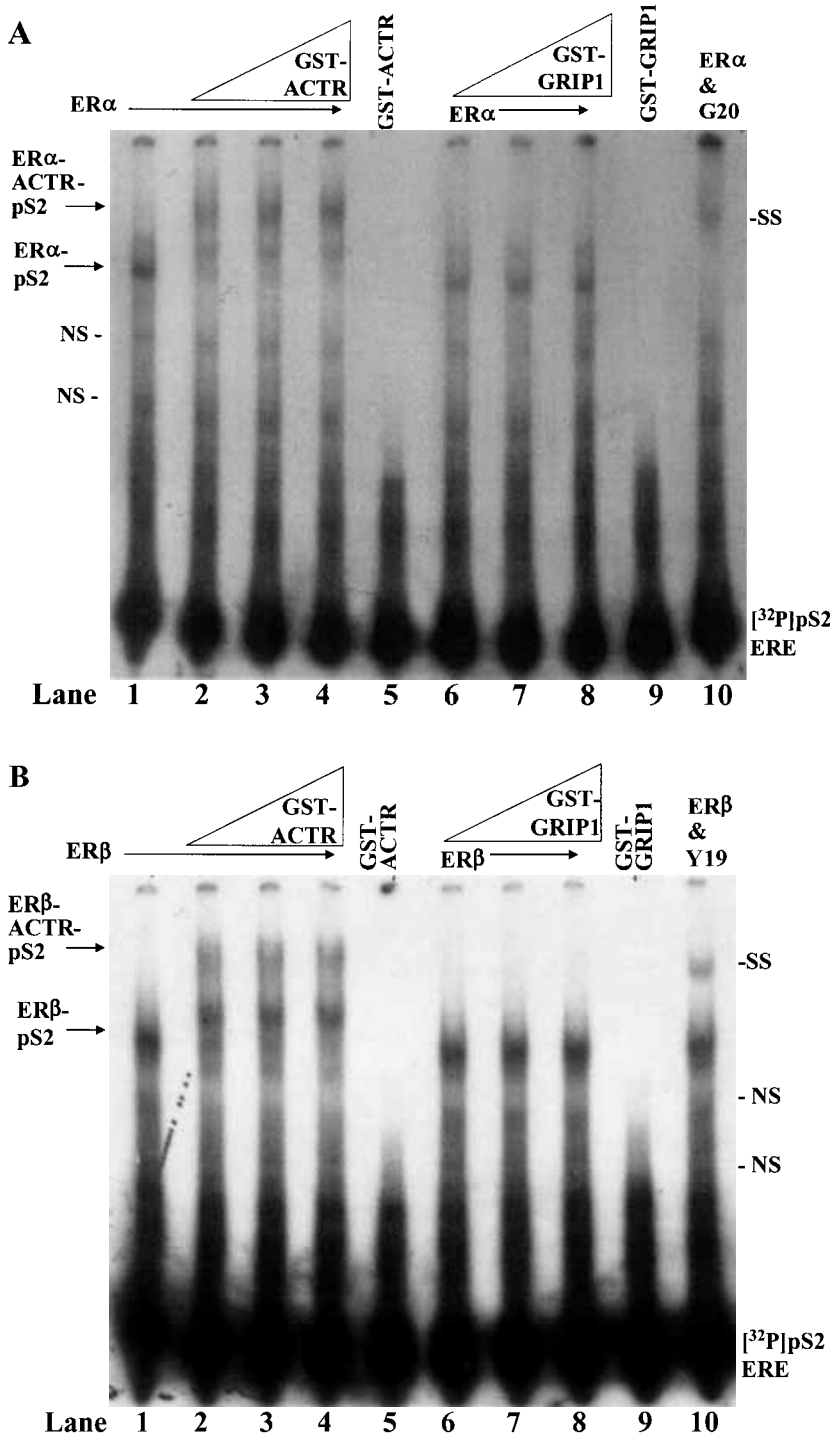
ERE correlates with E_2-induced transcriptional activity (Klinge 2001), there is not a correlation between ERE occupancy (K_d) and the transcriptional response to the tested coactivators, coregulators, and corepressors in transfected CHO-K1 cells. Thus, the effects of coregulatory proteins on E_2-activated transcription is not governed solely by the affinity of ER-ERE interaction, but we suggest reflects ERE-mediated alterations in ER conformation (Klinge *et al.* 2001, Ramsey & Klinge 2001, Yi *et al.* 2002) that we propose impact ER-coactivator interaction. Hence, although we are unable to compose a simple rule or set of rules that will predict how an ERE sequence impacts ER-coactivator interaction in transfected cells, our data clearly demonstrated that variations within the core ERE sequence regulate coactivator interaction with liganded ER. An important difference between our study and most previous studies (reviewed in Klinge 2003) is that we subtracted the effect of each coregulator on the ligand-independent (basal) activity of ERα or ERβ for each ERE. Thus, the findings in Table 1 summarize how coregulators impact E_2-dependent ERα and ERβ transcriptional activity from different EREs. We suggest that small changes with individual coactivators may be physiologically significant when combined with the other coregulators in the cell. For example, a 50–100% increase in transcription of the transcription factors Fos or PR could effectively double the amount of Fos and PR proteins. Doubling PR expression subsequently could have a synergistic effect on downstream gene targets.

Our results have shown that the length of the ERE palindrome differentially impacts coactivator activity for ERα and ERβ. Of the EREs examined in this study, EREc38 has the longest (17 bp) perfectly palindromic ERE, binds ERα and ERβ with highest affinity (Table 1), gives the highest E_2-induced transcription (Fig. 1), and shows the least effect of coactivators on E_2-induced ERα or ERβ activity. SRC-1 was the only coactivator that stimulated E_2-induced ERα but not ERβ transcription from EREc38. In contrast, SRC-1 had no effect on E_2-ERα activity on EREc13. Hence, the length of the ERE palindrome and the presence of the conserved 5′ AT-rich region found in the vit A2 gene appear to enhance ERα-SRC-1 functional interaction in this transient transfection assay.

The pS2 ERE showed the most ligand-dependent ER responsiveness to the coactivators tested. Based on a report showing low interaction of ERα-pS2 with SRC-1 and ACTR-RID peptides *in vitro* (Hall *et al.* 2002), we predicted that SRC-1 and ACTR would have minimal impact on E_2-ERα activity on the pS2-ERE reporter. Indeed, SRC-1 enhanced E_2-ERβ, but not ERα transcription from the pS2-ERE. On the other hand, ACTR stimulated E_2-ERα, but not ERβ transcription from the pS2 ERE, despite the interaction of GST-ACTR with both ERα and ERβ bound to the pS2 ERE *in vitro* (Fig. 8). This difference from the aforementioned predication (Hall *et al.* 2002) suggests that amino acids in addition to the RID in ACTR impact transactivation by ERα. This suggestion agrees with data showing that the three NR boxes from SRC-1, GRIP1, and ACTR have different affinities for E_2-occupied ERα and ERβ *in vitro* (Wong *et al.* 2001). Our cotransfection data are also in agreement with the observation that less GRIP1 was retained by ERα bound to the pS2 ERE versus the vit A2 ERE *in vitro* (Wood *et al.* 2001).

As anticipated from the discussion above, data from our EMSA experiments using GST-RIDs did not fully correlate with results from the transient transfection assays. These divergent results are most likely due to the use of a single RID domain in the EMSA experiments and the use of full-length proteins in the transient transfection assays. Our EMSA data are in agreement with time-resolved fluorescence resonance energy transfer (FRET) experiments showing no interaction between ERα and the first RID of SRC-1 and minimal recruitment to ERβ *in vitro* (Bramlett *et al.* 2001). Similarly, our EMSAs and the FRET studies (Bramlett *et al.* 2001) indicate that the first RID of ACTR interacts with both ERα and ERβ. Further experiments are necessary to determine how full-length coactivators influence ER-ERE binding affinity.

Results from scintillation proximity and mammalian two-hybrid assays showed that the ligand binding domain (LBD) of ERβ interacts with the RIDs of p160 coactivators in the following order of affinity (high–low): GRIP1>ACTR>SRC-1>>>p300/CBP (Northrop *et al.* 2000). These data suggest that E_2-ERβ transcription would be expected to be stimulated most by GRIP1, less by ACTR and SRC-1, and the least with CBP. On the other hand, intact ERβ interacted with comparable affinity with the immobilized NR boxes of SRC-1,



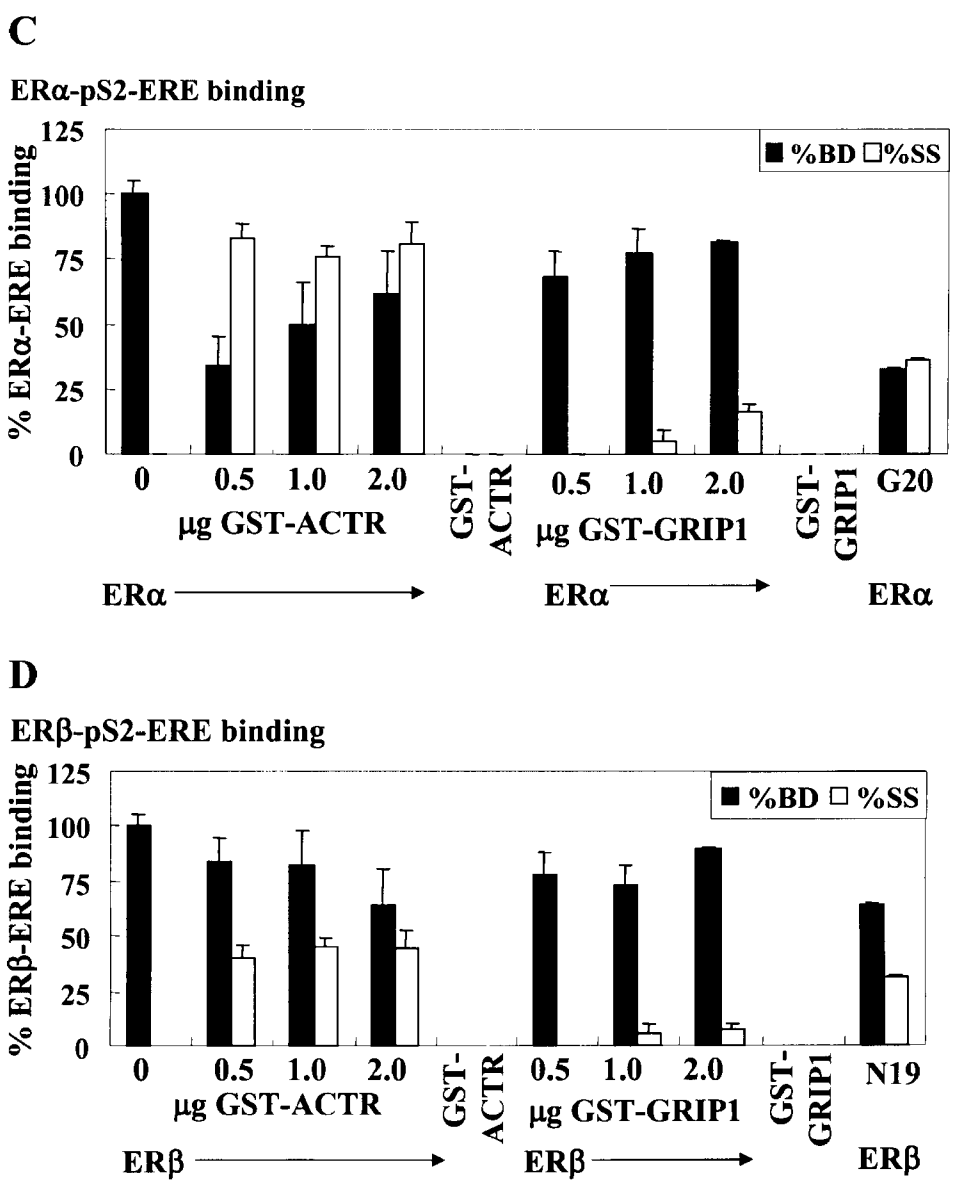
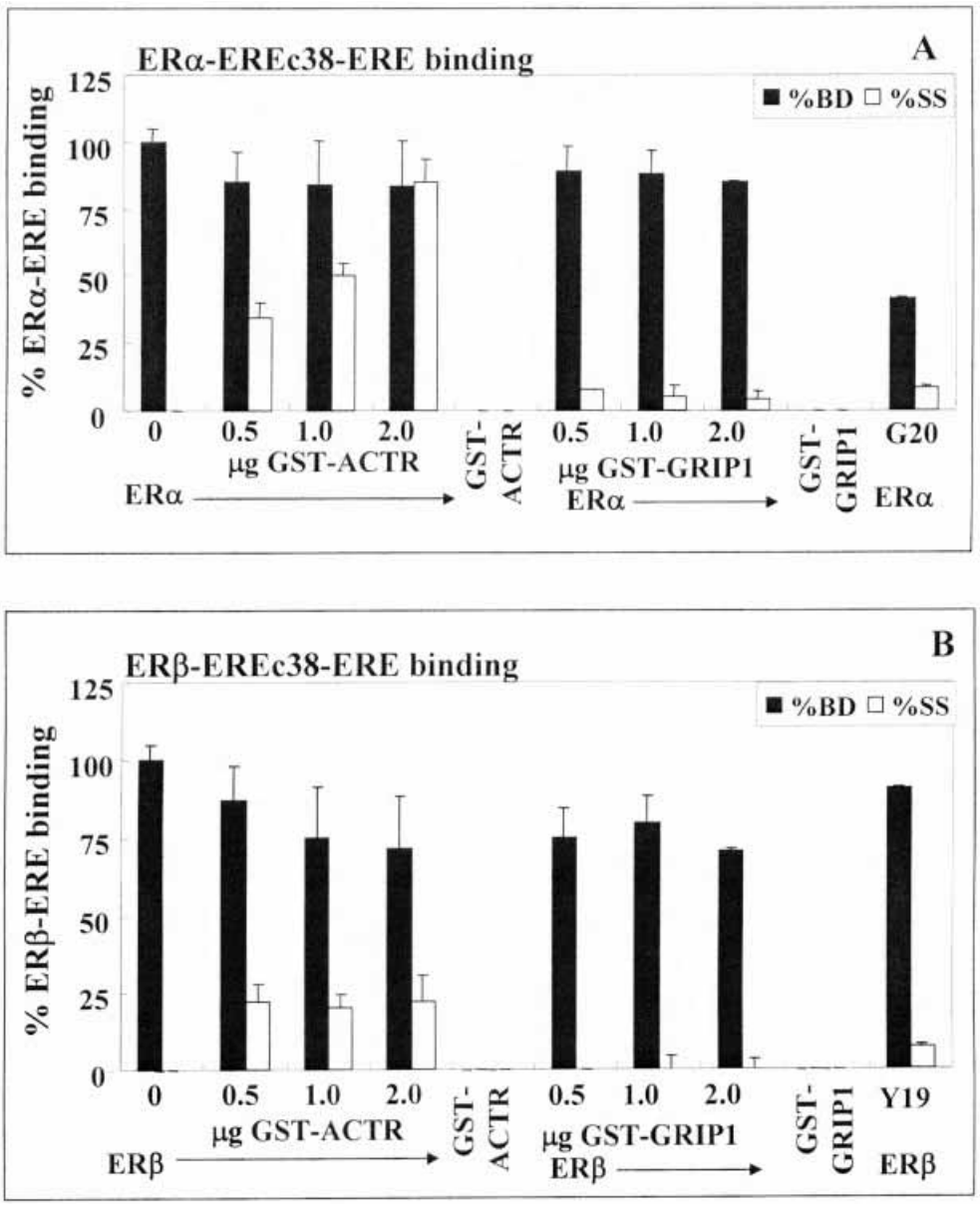


Figure 8 The RID of ACTR, but not GRIP1, interacts with ERα and ERβ bound to the pS2 ERE *in vitro*. (A) Human ERα was incubated with [^{32}P]pS2-ERE as described in Materials and methods. GST-ACTR or GST-GRIP1 (0·5, 1, or 2 μg) were added to ERα and [^{32}P]pS2-ERE in lanes 2–4 and 6–7 respectively, as indicated. GST-ACTR and GST-GRIP alone did not bind pS2-ERE (lanes 5 and 9). Lane 10 included 1 μl ERα-specific antibody G20. (B) Rat ERβ was incubated with [^{32}P]pS2-ERE as described in Materials and methods. GST-ACTR or GST-GRIP1 (0·5, 1, or 2 μg) were added to ERβ and [^{32}P]pS2-ERE in lanes 2–4 and 6–7 respectively, as indicated. GST-ACTR and GST-GRIP alone did not bind pS2-ERE (lanes 5 and 9). Lane 10 included 1 μl ERβ-specific antibody Y-19. NS indicates non-specific binding of baculovirus proteins to the ERE; SS indicates the super-shifted complex formed between the ER antibody and the ER-pS2 complex. This autoradiograph is representative of two independent EMSA experiments for both ERα and ERβ showing similar results. (C and D) EMSA data were quantitated as described in Materials and methods. Values are the % of (C) ERα or (D) ERβ binding in the absence of added coregulator or antibody. %BD=ER-ERE bound; %SS=ER-ERE supershifted with the added GST fusion protein or antibody. Values are the average of two separate experiments±S.D.



GRIP1, and ACTR *in vitro* (Wong *et al.* 2001). Hence, the length of the ERβ used influences coactivator interaction *in vitro*. In contrast to these *in vitro* results, we observed that GRIP1 stimulated E_2-ERβ transcription only from the pS2 ERE. One possible explanation of these results is that GRIP1 requires a cellular cofactor(s) for the other EREs that is not expressed in CHO-K1 cells. However, GRIP1 did not enhance E_2-ERβ activity on either EREc38 or pS2-ERE in HEC-1A human endometrial cancer cells (C M Klinge, unpublished data), indicating that CHO-K1 are not unique in this response. Cells that express endogenous ERβ should be tested to further evaluate the impact of GRIP1 on ERβ activity.

ERα interacts functionally with p300 and CBP (Shibata *et al.* 1997) which are 'cointegrators' because they form complexes with TBP and a variety of activator proteins (McKenna *et al.* 1999). CBP stimulated unliganded ERα and ERβ transcription at all EREs except PR1148. The finding that CBP enhanced E_2-ERα transcription only from the

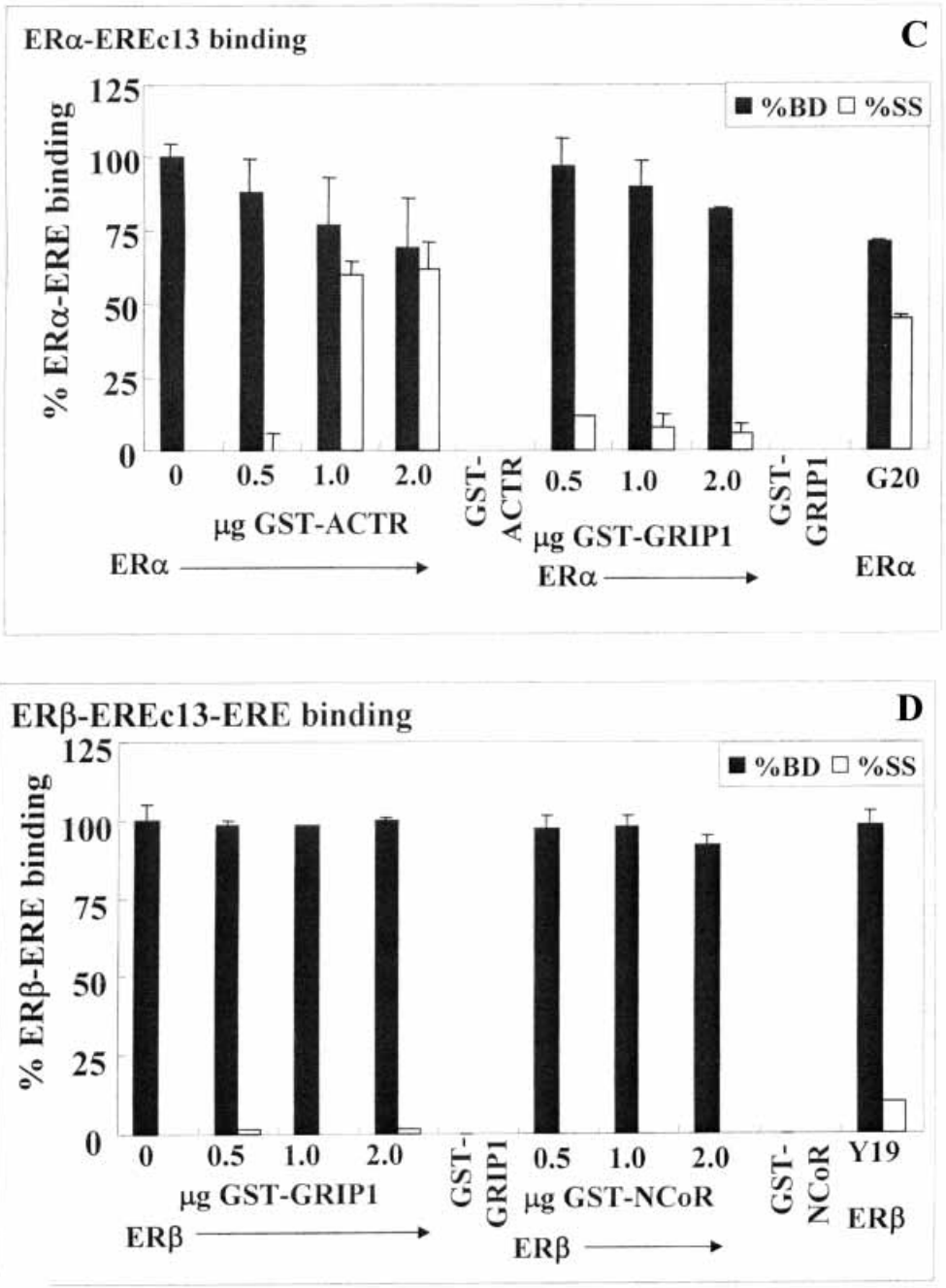


Figure 9 Effect of GST-coregulators on ER-ERE binding *in vitro*. EMSA data were quantitated as described in Materials and methods. Values are the % of (A and C) ERα or (B and D) ERβ binding to (A and B) EREc38 or (C and D) EREc13 in the absence of added coregulator or antibody. %BD=ER-ERE bound; %SS=ER-ERE supershifted with the added GST fusion protein or antibody. Values are the average of two to three separate experiments±S.E.M.

Table 3 Comparison of the effect of the indicated coregulator on the transcriptional activity E_2-ERα or -ERβ with the effect of the GST-RID of that coregulator on ERα or ERβ interaction with that of ERE *in vitro* in EMSA. Data are the summary of transient transfection assays shown in Figs 3–5 and three separate EMSA gels, examples of which are shown in Fig. 8, for each combination of ER-ERE and GST-RID

	E_2-ERα transcriptional activity	**EMSA E_2-ERα**	**E_2-ERβ transcriptional activity**	**EMSA E_2-ERβ**
ERE sequence				
EREc38	SRC-1 (stimulation) GRIP1 or ACTR (no effect)	No effect of SRC-1 or GRIP1*; ACTR supershifted the ERα-EREc38 complex	SRC-1, GRIP1, or ACTR (no effect) NCoR (stimulation)	No effect of SCR-1* or GRIP1*; ACTR supershifted the ERβ-EREc38 complex; no effect of NCoR
EREc13	SRC-1 (no effect) GRIP1 (stimulation) ACTR (stimulation)	No effect of SRC-1 or GRIP1; ACTR* supershifted the ERα-EREc13 complex	GRIP1 (no effect) NCoR (stimulation)	No effect of GRIP1* or NCoR
pS2	GRIP1 (no effect) ACTR (stimulation)	No effect of GRIP1*; ACTR* supershifted the ERα-pS2-ERE complex	GRIP1 (stimulation) ACTR (no effect)	No effect of GRIP1; ACTR supershifted the ERβ-pS2-ERE complex
PR1148	SRC-1 (stimulation) GRIP1 (no effect)	No effect of SRC1 or GRIP1*	GRIP1 (no effect) NCoR (stimulation)	No effect of GRIP1* or NCoR
Fos-1211	SRC1 (no effect) GRIP1 (stimulation)	No effect of SRC-1* or GRIP1	GRIP1 (no effect) NCoR (inhibition)	No effect of GRIP1 or NCoR

*Agreement between transient transfection and EMSA results.

PR1148 ERE is consistent with experiments showing that CBP shows weaker interaction with the ERα LBD than SRC-1 *in vitro* (Heery *et al.* 2001). In contrast to our ERα findings, CBP enhanced E_2-ERβ transcription from pS2 and PR1148 EREs (Table 1). Ours is the first examination of the impact of CBP on ligand-dependent ERβ-induced transcription. We conclude that CBP interacts differently with ERα and ERβ and that the ERE sequence impacts this interaction.

SRA is a unique ER coactivator because it functions as an RNA molecule *in vivo* and it enhances ERα transactivation through AF-1 (Lanz *et al.* 1999). Ours is the first study to compare the activity of SRA with ERα and ERβ on natural EREs. Although a recent study reported that SRA enhanced the activities of E_2-ERα and -ERβ on a two-tandem vit A2 ERE-luciferase reporter in COS-1 or HEK-293 cells without affecting basal activity (Deblois & Giguere 2003), we observed that SRA stimulated basal, unliganded ERα and ERβ activity on all EREs with the exception of ERβ on the pS2 ERE. SRA showed the greatest activity with unliganded ERα and ERβ on the Fos-1211 ERE. This finding warrants further investigation in future studies. Data showing that SRA interacts with p72 and p68 coactivators, which bind GRIP1 but do not interact with or activate ERβ (Watanabe *et al.* 2001), imply that SRA may not stimulate ERβ transcription. Our data are in agreement with this prediction: SRA did not enhance E_2-ERβ transcription with the exception of the pS2 ERE, indicating that the ERE sequence impacts SRA-ERβ interaction. Since SRA forms a complex with SRC-1 (Lanz *et al.* 1999), we evaluated the combined effect of the p160 coactivators and SRA on E_2-induced ERα and ERβ activity on different EREs. Our results indicated that

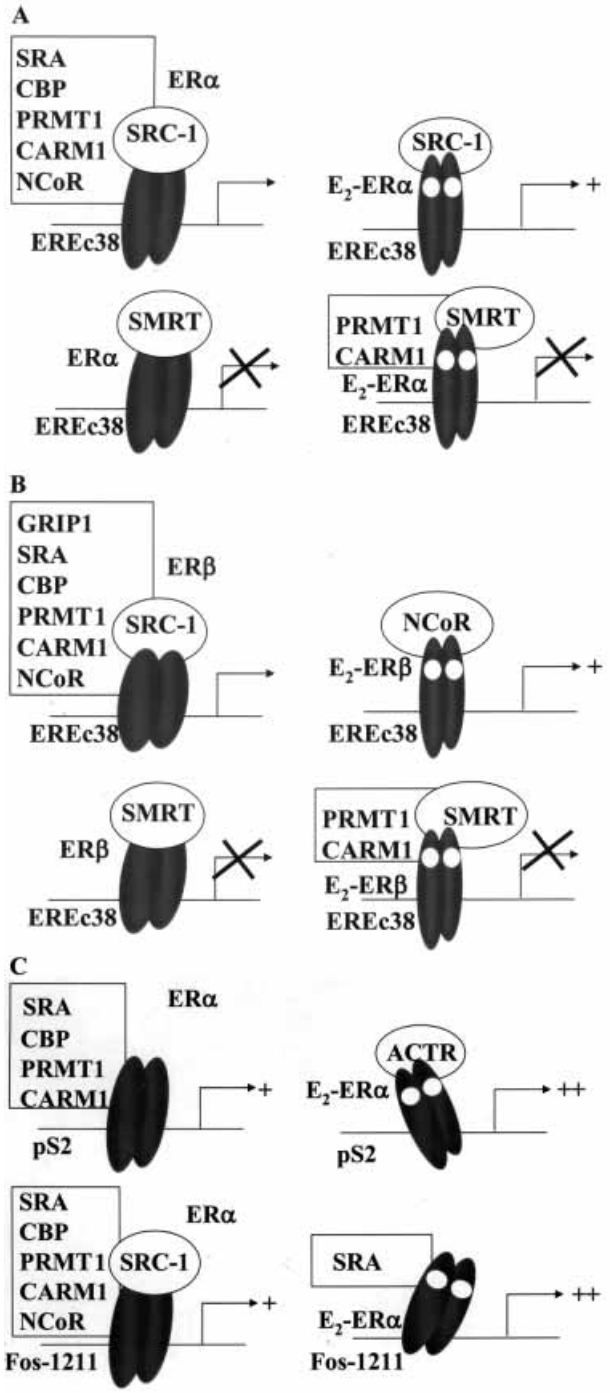


Figure 10 Model for ERE sequence-, ER subtype-, coregulator- and ligand state-dependent transcription by ER. Coactivators and corepressors regulate ERα and ERβ transcriptional activity depending on different conformations of the receptors in their unliganded versus E_2-occupied as they bind to EREs from different genes. Please see Discussion for further details.

both ER subtype and DNA sequence impact SRA activity.

CARM1 and PRMT1 interact with p160 family coactivators and stimulate NR transcription (Chen *et al.* 2000, Koh *et al.* 2001). PRMT1 stimulated unliganded ERα and ERβ activity on all EREs. CARM1 differed from PRMT1 in that it did not stimulate unliganded ERα and ERβ on the PR1148 and Fos-1211 EREs. The only E_2-dependent stimulatory activity of CARM1 was with E_2-ERβ on the pS2 ERE. It will be important to determine if the ERE sequence impacts the methyltransferase activity of PRMT1 and CARM1.

In GST pull-down assays, corepressors NCoR and SMRT interact with ERα in a ligand-independent manner (Smith *et al.* 1997, Lavinsky *et al.* 1998). Chromatin immunoprecipitation experiments revealed that NCoR interacts with ERα in the promoters of the c-Myc and cathepsin D genes in MCF-7 breast cancer cells treated with tamoxifen or raloxifene (Shang & Brown 2002), indicating that these corepressors play a role in transcriptional silencing. Here we noted that NCoR increased unliganded ERα and ERβ activity from all but the pS2 and PR1148 EREs. Differences between native chromatin and partial chromatin structure on transfected reporter constructs may be responsible for this difference. The mechanism of stimulation of ER basal activity by NCoR is unclear; however, NCoR and SMRT interacted directly with ACTR, SRC-1, and GRIP1 *in vitro* and in transfected cells (Li *et al.* 2002). Further, NCoR enhanced unliganded TRβ-ACTR interaction (Li *et al.* 2002). Whether NCoR facilitates ER-endogenous ACTR interaction in the absence of ligand is unknown. Further studies are needed to address the role of NCoR in fine-tuning transcriptional repression versus activation by ERα and ERβ.

In mammalian two-hybrid assays, NCoR had no effect on E_2-activated ERα or ERβ reporter expression (Zhang *et al.* 2000). In contrast, NCoR inhibited E_2-induced ERα transcription only from the pS2 and Fos-1211 EREs in our cell-based assays. Therefore, the ERE sequence impacts NCoR-mediated transcriptional repression from a transfected plasmid. SMRT reduced basal and 4-OHT-stimulated reporter gene activity in HepG2 cells transfected with ERα, but had no effect on E_2-activated reporter activity (Smith *et al.* 1997).

Similarly, we found that SMRT inhibited basal transcription by ERα or ERβ, but SMRT inhibited E_2-induced transcription for both ERα and ERβ, although the effect was more pronounced for ERα than ERβ. Notably, NCoR enhanced 4-OHT inhibition of ERα activity, but not ERβ activity whereas SMRT increased 4-OHT inhibition of ERβ, not ERα.

In summary, the data presented here have demonstrated for the first time that the nucleotide sequence of the ERE and its immediate flanking sequences from the native gene differentially impact ERα and ERβ transcription and the functional interaction of coregulatory proteins in transfected cells. In agreement with previous investigators we conclude that the ERE nucleotide sequence impacts physical (Wood *et al.* 1998, 2001, Loven *et al.* 2001*a*, Hall *et al.* 2002) and functional interaction of ERα and ERβ with coregulators. These data are in agreement with our previous studies and those of other investigators showing that DNA is an allosteric modulator of ER action (reviewed in Klinge 2003). This allows gene-specific recruitment of coregulators to ERα and ERβ which, along with cell-specific ratios of coregulator expression, results in tissue-specific gene transcriptional responses to E_2 *in vivo*.

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