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Cofactor Dynamics and Sufficiency in Estrogen Receptor–Regulated Transcription

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Summary

Many cofactors bind the hormone-activated estrogen receptor (ER), yet the specific regulators of endogenous ER-mediated gene transcription are unknown. Using chromatin immunoprecipitation (ChIP), we find that ER and a number of coactivators rapidly associate with estrogen responsive promoters following estrogen treatment in a cyclic fashion that is not predicted by current models of hormone activation. Cycles of ER complex assembly are followed by transcription. In contrast, the anti-estrogen tamoxifen (TAM) recruits corepressors but not coactivators. Using a genetic approach, we show that recruitment of the p160 class of coactivators is sufficient for gene activation and for the growth stimulatory actions of estrogen in breast cancer supporting a model in which ER cofactors play unique roles in estrogen signaling.

Introduction

Estrogen plays an important role both in reproductive physiology and in numerous human disease states, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer's disease. The biological actions of estrogen are mediated by the products of two genes within the nuclear receptor family, estrogen receptor (ER) α and β. Current models of ER action suggest that it modulates the rate of transcription initiation through interactions with the basal transcription machinery and through alterations in the state of chromatin organization at the promoter of target genes via the recruitment of a variety of coactivators.

The assortment of coactivator proteins implicated in estrogen signaling includes three distinct but related p160 family members, SRC-1, TIF2 or GRIP-1, and AIB1 (also referred to as ACTR, RAC3, pCIP, or NCoA-3) (Onate et al., 1995; Anzick et al., 1997; Chen et al., 1997; Hong et al., 1997; Li et al., 1997; Torchia et al., 1997) and the histone acetylases CBP, p300 (Chakravarti et al., 1996; Hanstein et al., 1996) and the p300/CBP-associated factor, pCAF (Blanco et al., 1998). Coactivators such as CBP, p300, pCAF, and possibly SRC1 and AIB1 possess intrinsic histone acetyltransferase (HAT) activities capable of modifying the chromatin organization of the target gene promoters. However, the participation of all of these proteins with a common enzymatic activity in ER transactivation raises the question of functional redundancy. In addition a distinct multiprotein complex first found to be involved in thyroid hormone receptor (TR) and vitamin D receptor (VDR) signaling (Fondell et al., 1996; Rachez et al., 2000) has also been implicated recently in ER action through an interaction with its PBP/TRAP220/DRIP205 subunit (hereafter PBP) (Burakov et al., 2000).

Much of our current knowledge regarding the involvement of these proteins in nuclear receptor signaling is based on their ability to bind liganded-receptors in vitro and enhance transcriptional activation in transfection experiments. Although the participation of so many protein factors in ER-mediated gene transactivation undoubtedly reflects the complexity of the transcription in eukaryotes, it also raises the question of which if any of these coactivators are necessary and/or sufficient for the transcriptional activation of ER in vivo. In addition, the transcriptional dynamics of ER action are also not well understood. The traditional view that activators such as ER bind to a response element in the promoter of a target gene and remain associated for as long as the stimulus is present (Hahn, 1998; Berk, 1999) is inconsistent with a recent report that the association of ER and AIB1 is a transient process that is disrupted by acetylation of AIB1 by CBP/p300 (Chen et al., 1999). This raises the question of whether the ER complex might cycle on and off the target gene promoters.

We have addressed these critical questions under biologically relevant conditions through the study of endogenous ERα and cofactors in breast cancer cells. Using chromatin immunoprecipitation (ChIP) we find that in response to estrogen native ER transcription complexes are stimulated to assemble on target promoters in a cyclic fashion. These experiments demonstrate differential roles for the various coregulators in the assembly of ER transcription complexes in vivo. In addition we demonstrate that TAM-bound ER recruits corepressors but not coactivators to target promoters suggesting that the cellular responses to a selective ER modulator (SERM) such as TAM may reflect the balance between coactivators and corepressors present in different cells. Finally, using a p160 coactivator that binds ER with reversed ligand specificity, we show that recruitment of this class of coactivator is sufficient for gene activation as well as estrogen-dependent cell cycle progression.

Results

Estrogen Induces Occupancy of Estrogen Target Gene Promoters by ERα and p160 Coactivators

To investigate the assembly of the ER transcription complex, we first examined the recruitment of ERα and p160

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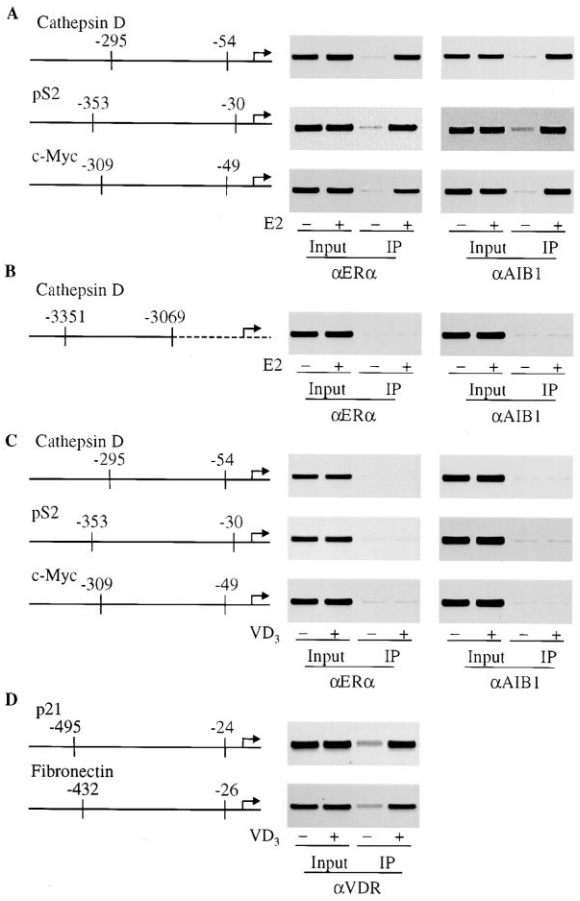

Figure 1. The Recruitment of ERα and AIB1 to the Promoters of Estrogen-Responsive Genes

(A) Soluble chromatin was prepared from MCF-7 cells treated with E2 for 45 min and immunoprecipitated (IP) with antibodies against ERα (αERα) or against AIB1 (αAIB1). The final DNA extractions were amplified using pairs of primers that cover the regions of cathepsin D, pS2 and c-Myc gene promoters as indicated. (B) The distal region of the cathepsin D gene promoter was examined for the presence of ERα and AIB1. (C) MCF-7 cells were treated with 1,25-$(OH)_2D_3$ (VD_3) for 45 min and the cathepsin D, pS2, and c-Myc gene promoters were examined for the occupancy by ERα or AIB1 using the same antibodies and primers as in (A). (D) MCF-7 cells were treated with 1,25-$(OH)_2D_3$ (VD_3) for 45 min and the occupancy of the vitamin D receptor (VDR) on the p21 and fibronectin gene promoters was examined.

coactivators to the promoters of endogenous estrogen-responsive target genes following estrogen treatment. The estrogen-dependent human breast cancer cell line MCF-7 was used for these studies as there are several well-characterized estrogen target genes in these cells. Three estrogen target genes, cathepsin D (CATD), pS2, and c-Myc (Dubik and Shiu, 1992; Augereau et al., 1994; Giamarchi et al., 1999) were examined. MCF-7 cells were grown in the absence of estrogen for at least three days followed by either no treatment or treatment with saturating levels of 17β-estradiol (E2) for 45 min. The status of the endogenous transcription complexes present on the estrogen responsive regions of these promoters was determined using chromatin immunoprecipitation (ChIP). The presence of the specific promoters in the chromatin immunoprecipitates was analyzed by semiquantitative PCR using specific pairs of primers spanning the estrogen responsive regions in the three promoters. As shown in Figures 1A, treatment with E2 induced a dramatic increase in the occupancy by both ERα and AIB1 of the CATD, pS2 and c-Myc gene promoters. Similar promoter occupancy by the other p160 family members SRC-1 and GRIP1 was also observed (data not shown). As expected, given the ~500 bp to ~2000 bp size of the DNA fragments produced by sonication in these experiments, PCR analysis did not detect any significant increase in ERα or AIB1 occupancy of a region ~3 kb upstream of the CATD promoter (Figure 1B). In addition, treatment of MCF-7 cells under the same conditions with 1,25-$(OH)_2D_3$ (VD_3) failed to induce any increase in ERα or AIB1 association with these promoters (Figure 1C), although VD_3 treatment did result in an increased occupancy of the VD_3 receptor (VDR) on the promoters of VD_3 responsive p21 (Liu et al., 1996; Verlinden et al., 1998) and fibronectin (Polly et al., 1996) genes (Figure 1D).

Dynamics of ER Transcription Complex Assembly

Having shown that estrogen is able to induce occupancy of responsive promoters by ERα and AIB1, we sought to understand the precise order and timing of complex assembly using ChIP. Strikingly, ERα is recruited to the CATD promoter within 15 min following the addition of E2 (Figure 2A). ERα promoter occupancy peaks at 30–45 min and returns to baseline by 75 min. To further validate these findings, we designed a highly quantitative ChIP assay using real-time PCR and an ABI PRISM 7700 Sequence Detector (Perkin-Elmer). Quantitative ChIP was performed to determine the relative levels of CATD promoter occupancy by ERα following treatment of MCF-7 cells with E2. The measured level of CATD promoter occupied by ERα increased ~50-fold within 15 min following the addition of E2. Maximal induction of promoter occupancy of greater than 100-fold was detected at 30 min and this returned almost to baseline at later times. Thus the differences observed by ChIP in Figure 2A reflect very significant quantitative changes in promoter occupancy and confirm that ERα cycles onto and off of the CATD promoter in response to E2. In order to rule out epitope masking as an alternate explanation for the apparent cycling of ERα, we used multiple monoclonal antibodies directed toward different ERα epitopes in the ChIP assay. Three different monoclonal antibodies gave almost identical patterns of ERα association with the CATD promoter suggesting that epitope masking is an unlikely explanation for the observed cycling (data not shown).

We next sought to determine the participation and timing of association of the various coactivator proteins in the formation of the ERα transcription complex, and the relationship of this to the state of histone acetylation and the onset of gene transcription. As was observed for ERα, the coactivators AIB1, PBP, and p300 all rapidly and transiently associate with the CATD promoter as significant CATD promoter occupancy is observed within 15–30 min following the addition of E2. Concurrent with this first wave of factor association, histones associated with the CATD promoter are acetylated. This is closely followed by the association of RNA pol II. CBP

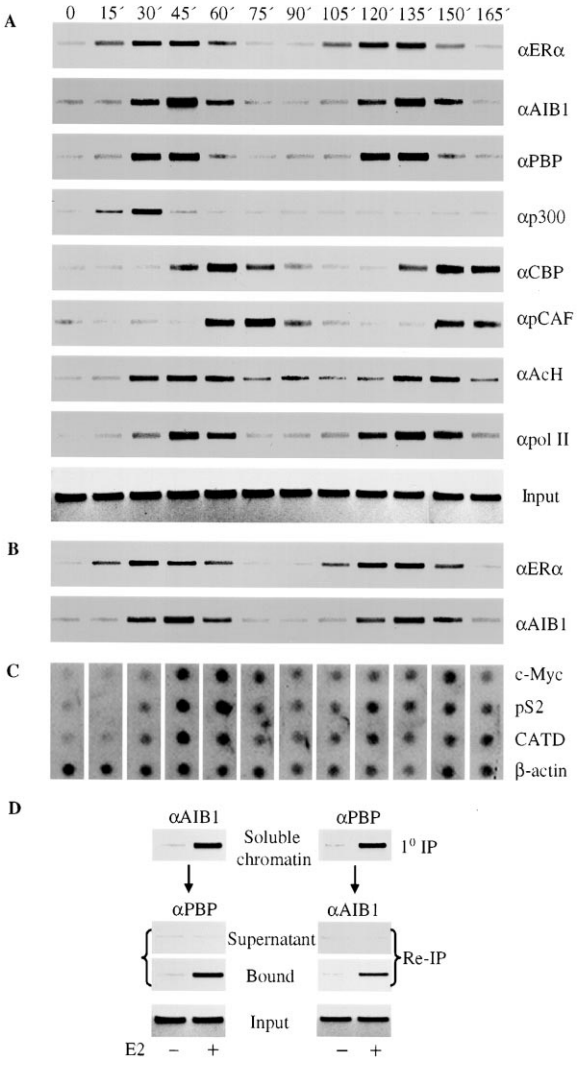

Figure 2. The Dynamics of ERα Transcription Complex Assembly

(A) Occupancy of the cathepsin D (CATD) promoter by ERα, different coactivators, acetylated histones (AcH) and RNA polymerase II (pol II) at different times as measured by ChIP. (B) Occupancy of the pS2 promoter by ERα and AIB1 as measured by ChIP. (C) Nuclear run-on analysis of the expression of c-Myc, pS2, CATD, and β-actin mRNA in MCF-7 cells treated with E2 for various times. (D) ChIP Re-IP to examine whether the ERα-PBP complex and ERα-AIB1 complex are assembled on the same promoters. Soluble chromatin was prepared from MCF-7 cells treated with E2 for 40 min and divided into two aliquots. One aliquot was first immunoprecipitated with antibodies against AIB1 (1^0 IP). The supernatant was collected and reimmunoprecipitated with antibodies against PBP (Supernatant Re-IP). The other aliquot was first immunoprecipitated with antibodies against PBP (1^0 IP) followed by reimmunoprecipitation with antibodies against AIB1. Similar reciprocal Re-IPs were also performed on complexes eluted from the 1^0 IPs (Bound Re-IP).

and pCAF also become transiently associated with the same region of the CATD promoter but only beginning at about 45 min following the onset of E2 stimulation. This is a time when association of the first wave of factors is already falling suggesting that CBP and pCAF function at a distinct step in the process of ERα-mediated activation.

Notably, the second cycle of promoter occupancy beginning about 100 min following estrogen stimulation differs somewhat from the first. While ERα, AIB1, PBP, CBP, pCAF all assemble in the same order and with the same timing as in the first cycle, p300 is not detected in the second cycle of complex assembly. Interestingly, while a second increase in the level of acetylated histones associated with the CATD promoter is detected during the second cycle, this is from a baseline level that remains elevated between the first and second cycle of complex assembly.

In order to confirm that the repeated cycling of the ERα complex was not unique to the CATD promoter in MCF-7 cells, we examined in detail the association of ERα and AIB1 with the pS2 promoter (Figure 2B). As was seen on the CATD promoter, both ERα and AIB1 repeatedly cycle onto and off of the pS2 promoter with very similar dynamics. This suggests that the cyclic nature of ERα complex assembly may be a general property of ERα-regulated genes. In addition, to determine whether the cyclic nature of the recruitment of the ER transcription complex to the CATD promoter is restricted to MCF-7 breast cancer cells, similar time courses were performed in ECC-1 endometrial cancer cells. Like MCF-7, ECC-1 cells express ERα and are E2 responsive for CATD expression and growth (Castro-Rivera et al., 1999). A very similar pattern of ERα and AIB1 recruitment to the CATD promoter was seen in ECC-1 as was seen in MCF-7 (data not shown). Whether the details of the factors involved and the timing of their assembly differ in a promoter- and/or cell type–specific manner is an intriguing possibility that remains to be determined.

To assess when the ERα transcription complex becomes competent for gene activation, we examined whether the repeated cycles of complex assembly are followed by transcription. Nuclear run-on assays were performed on the c-Myc, pS2, and CATD genes following estrogen stimulation of MCF-7 cells (Figure 2C). Significant transcription is evident after 45 min of estrogen stimulation. This follows the assembly of ERα, PBP, AIB1, p300, and pol II on the promoter and precedes the association of CBP and pCAF. Interestingly, as is seen with assembly of ERα and its associated cofactors on the promoter, transcription is also cyclic. These results confirm that the cyclic assembly of the ERα complex on the promoter is followed by cycles of transcription.

Additionally, the time course of coactivator recruitment reveals important aspects of the function of two distinct coactivator complexes. PBP, the protein that anchors the DRIP/TRAP complex to ERα and to other nuclear receptors (Rachez et al., 1998, 1999, 2000; Burakov et al., 2000) is recruited to the CATD promoter at the same time as ERα and AIB1. This result rules out a sequential model and supports either a combinatorial model in which the PBP and AIB1 containing complexes act simultaneously on the very same CATD promoters or a parallel model in which the two complexes are being recruited to distinct subsets of CATD promoters on different alleles or in different cells. In order to test whether a combinatorial or a parallel model applies, we performed a serial ChIP experiment (Figure 2D). For this we divided the soluble chromatin derived from E2-treated or untreated cells into two aliquots. One was immunopre-

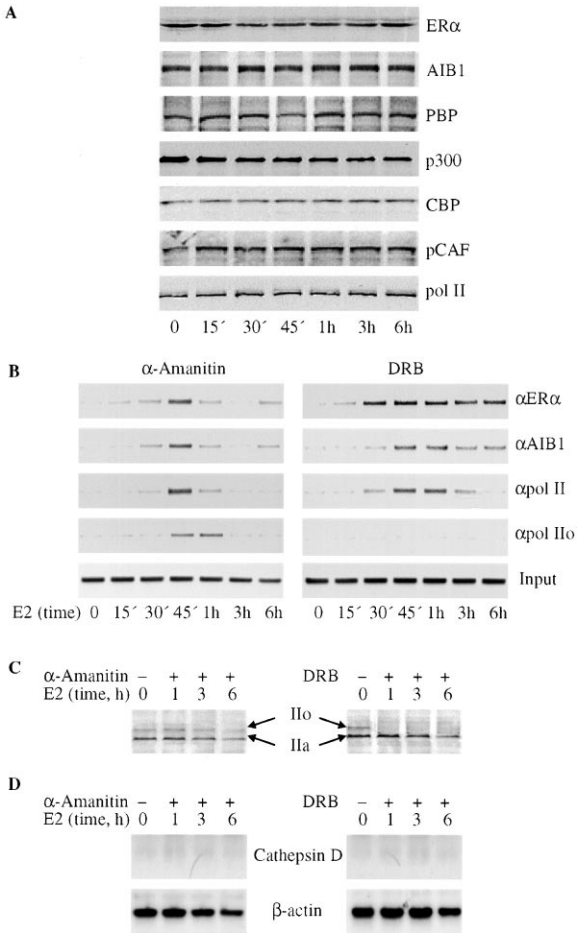

Figure 3. Mechanism of ERα Transcription Complex Disassembly

(A) Protein levels of the components of the ERα transcription complex in MCF-7 cells at different times following estrogen treatment measured by Western blotting. (B) Recruitment patterns of ERα, AIB1, RNA polymerase II (pol II) and phosphorylated RNA polymerase II (pol IIo) on the cathepsin D promoter in MCF-7 cells treated with α-amanitin (left panel) or DRB (right panel) followed by E2 for different times. (C) Western blot analysis of the phosphorylation status of RNA polymerase II after treatment with α-amanitin (left panel) or DRB (right panel) followed by E2 for various times. Both underphosphorylated (IIa) and hyperphosphorylated (IIo) forms of the large subunit of RNA polymerase II are shown. (D) Inhibition of transcription by α-amanitin (left panel) and DRB (right panel) was confirmed by Northern blotting in MCF-7 cells for the expression of the CATD mRNA. β-actin serves as a loading control.

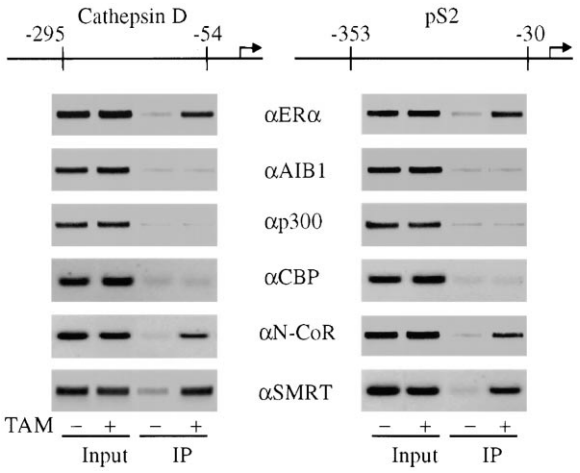

Figure 4. Promoter Occupancy by ERα and Cofactors Induced by Tamoxifen (TAM)

(A) ChIP demonstrates the promoter occupancy by ERα and the indicated cofactors on the CATD (left panel) and pS2 (right panel) promoters from MCF-7 cells treated with TAM.

cipitated with AIB1 antibodies followed by release of the immune complexes and reimmunoprecipitated (Re-IP) with PBP antibodies. The other was first immunoprecipitated with PBP antibodies followed by release and Re-IP with AIB1 antibodies. The same Re-IP was also performed on the unbound supernatant fractions from the primary immunoprecipitation. While both AIB1 antibodies and PBP antibodies were able to immunoprecipitate the CATD promoter after cells were treated with E2 (Figure 2D), subsequent supernatant Re-IPs with either PBP antibodies or AIB1 antibodies were unable to do so. On the other hand, subsequent Re-IPs of the eluted primary immunoprecipitates were able to bind the CATD promoter ("bound" in Figure 2D). These experiments support a model in which AIB1 and PBP act in a combinatorial fashion on the same ERα responsive promoter.

Phosphorylation of pol II Is Required for ER Complex Cycling

Our data show that the association of ERα and the other components of the transcription initiation complex with the promoter is cyclical. This raises the question of what events regulate the release of the complex from the promoter. We first examined whether the overall cellular levels of the factors change over the time course of estrogen stimulation. The levels of ERα and the other factors do not fluctuate significantly over the first hour of E2 treatment during which time they have cycled onto and off of the CATD promoter and stable levels are present for as long as 6 hr (Figure 3A).

To address whether release from the promoter occurs at a specific step during transcription initiation, two inhibitors of transcription, α-amanitin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were used. MCF-7 cells were treated with either 10 μg/ml of α-amanitin or 50 μM of DRB for 1 hr before the addition of E2, and CATD promoter occupancy was determined by ChIP for ERα, AIB1, and both total RNA pol II and its hyperphosphorylated IIo form (Figure 3B).

α-Amanitin is able to bind to the large subunit of RNA pol II (Kedinger et al., 1970; Lindell et al., 1970) and block the incorporation of new nucleotides into the nascent RNA chain (de Mercoyrol et al., 1989). Interestingly α-amanitin had no obvious effect on the pattern of CATD promoter occupancy by ERα, AIB1, and RNA pol II (Figure 3B, left panel) or the generation of the phosphorylated IIo form of pol II (Figures 3B and 3C, left panel) though CATD transcription was significantly inhibited by α-amanitin (Figure 3D, left panel). Thus, the assembly and subsequent release of the ERα transcription complex from the CATD promoter does not depend on the incorporation of nucleotides into the nascent RNA transcript.

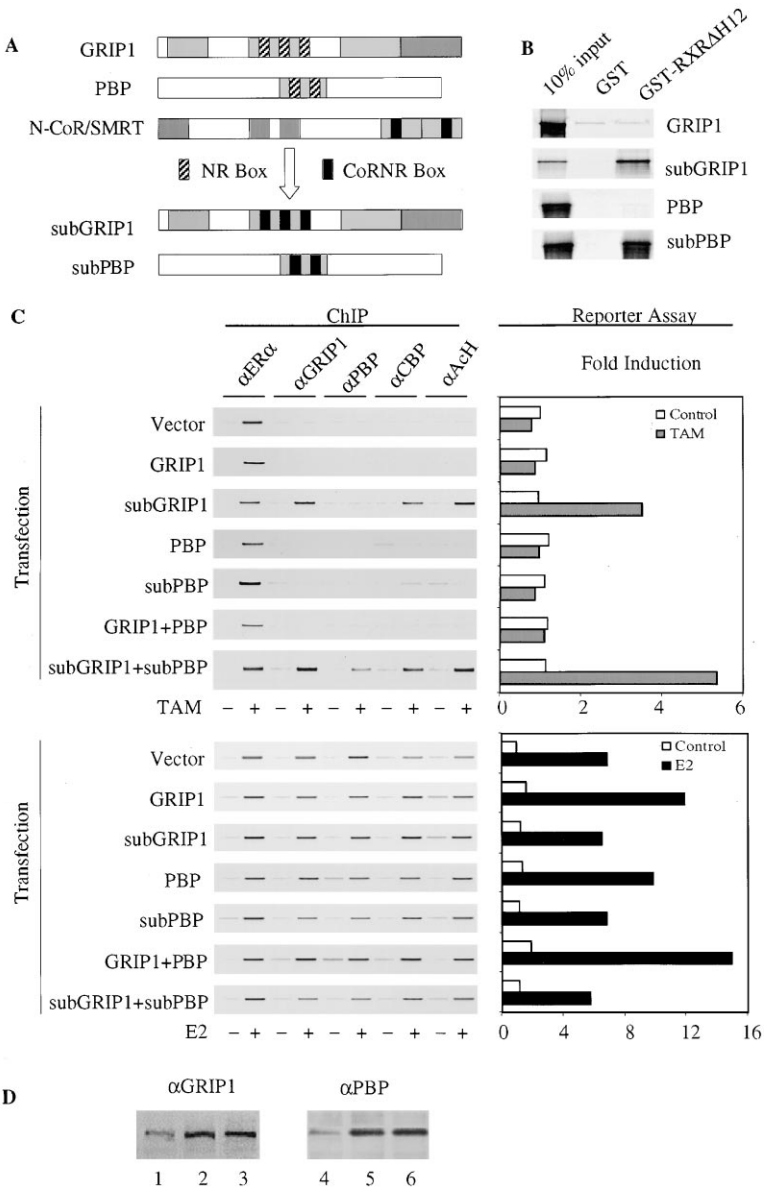

Figure 5. Central Role for p160 Proteins in the Assembly of the ERα Transcription Complex

(A) Diagram of the structure of subGRIP1 and subPBP. (B) Interaction of in vitro transcribed and translated GRIP1, PBP, subGRIP1, and subPBP with glutathione S-transferase (GST) or a GST fusion of helix 12 deleted RXR (GST-RXRΔH12) was assayed by GST pulldown in the absence of ligand. (C) ChIP (left panel) and reporter (right panel) assays were performed on MCF-7 cells transfected with the indicated expression plasmids and treated with TAM (upper panel) or E2 (lower panel). The occupancy of the CATD promoter by ERα, GRIP1, CBP, PBP, and acetylated histone (AcH) was examined. (D) Western blotting analysis of protein expression in MCF-7 cells transfected with GRIP1 (lane 2) or subGRIP1 (lane 3), or transfected with PBP (lane 5) or subPBP (lane 6). Lane 1 and lane 4 are vector transfection controls.

DRB acts to block transcription by inhibiting CDK7 (Yankulov et al., 1995) and CDK9 (Marshall et al., 1996), two kinases responsible for phosphorylating the RNA pol II large subunit C-terminal domain. In contrast to what we found with α-amanitin, DRB treatment led to the stabilization of ERα, AIB1, and RNA pol II on the CATD promoter for several hours (Figure 3B, right panel). As expected DRB also effectively blocked CATD transcription (Figure 3D, right panel) as well as the phosphorylation of pol II (Figures 3B and 3C, right panel). These results suggest that the release of the ERα transcription complex from the promoter requires the phosphorylation of the RNA pol II large subunit and are consistent with other data showing that the activity of RNA polymerase II is regulated by multisite phosphorylation on its C-terminal domain. Underphosphorylated RNA pol II C-terminal domain is believed to mediate multiple protein–protein interactions involved in the assembly of the preinitiation complex while the subsequent phosphorylation of the C-terminal domain contributes to the initiation of transcription and elongation of the primary transcript (Corden and Patturajan, 1997; Bentley, 1998). Taken together with the ability of the ERα transcription complex to be released from the promoter in the presence of α-amanitin, we conclude that promoter release occurs subsequent to RNA pol II C-terminal domain phosphorylation and prior to or concurrent with transcription initiation.

Tamoxifen Induces the Formation of an ER-Corepressor Complex

Tamoxifen (TAM) competes with E2 for ERα binding and induces a conformational change in which the recruitment of p160 coactivators is blocked (Halachmi et al., 1994; Brzozowski et al., 1997). In addition, while it functions as an antagonist in breast cancer cells such as MCF-7, in other tissues and on certain promoters TAM acts as a partial ERα agonist. This property has led TAM

to be viewed as the prototypical selective ER modulator or SERM. To investigate further the mechanisms underlying the activity of TAM-ERα, we examined the recruitment of coactivators or corepressors in MCF-7 cells after treatment with TAM (Figure 4). As was observed with E2, TAM treatment induced ERα occupancy of the CATD (Figure 4, left panel) and pS2 promoters (Figure 4, right panel). As expected, the TAM-ERα complex did not recruit p160 coactivators such as AIB1 nor CBP nor p300. In marked contrast, when we examined promoter occupancy by corepressors, we detected the recruitment of the nuclear receptor corepressors N-CoR and SMRT. These data show that in addition to inducing a conformational change in ERα that blocks coactivator recruitment, TAM is able to induce the recruitment of ERα and an associated corepressor complex to the promoter, suggesting TAM-ERα may be actively involved in gene repression. This also suggests that the ratio of agonism to antagonism seen with SERMs such as TAM may be influenced by the levels or activity of the corepressor complex.

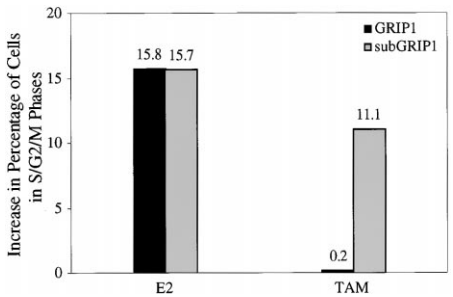

Figure 6. Induction of Cell Proliferation in MCF-7 Cells Expressing subGRIP1

MCF-7 cells were grown in DMEM supplemented with 10% charcoal-dextran-stripped fetal bovine serum for 24 hr and were cotransfected with a GFP expression plasmid together with either a GRIP1 or a subGRIP1 expression plasmid. Forty-eight hours after transfection, cells were treated with 100 nM E2 or 5 μM TAM or untreated for another 16 hr. Cells were then collected and analyzed by flow cytometry. The numbers indicate the increase in the percentage of GFP-positive cells that are in the S/G2/M phases of the cell cycle after 16 hr treatment with E2 or TAM.

p160 Proteins Play a Central Role in the Assembly and Activity of the ER Transcription Complex

PBP and the p160 coactivators utilize similar NR boxes that specifically recognize the agonist-bound conformation of ERα. The NR box is characterized by an LXXLL sequence flanked with a short stretch of amino- and carboxyl-terminal amino acids and is both necessary and sufficient for ligand-dependent interactions of p160 proteins and PBP with AF2 domains of nuclear receptors (Heery et al., 1997; Ding et al., 1998). Analogously, the two interaction domains of the nuclear receptor corepressors have been found to contain a conserved sequence referred to as the CoRNR box (Hu and Lazar, 1999) or as an LXXI/HIXXXI/L helix that recognizes the unliganded and repression competent form of thyroid and retinoid receptors (Nagy et al., 1999; Perissi et al., 1999). It has been suggested that the nuclear receptor AF-2 helix has evolved to discriminate between the NR box LXXLL helix in coactivators and the CoRNR box helix in the N-CoR/SMRT corepressors, permitting the ligand-dependent switch of nuclear receptor activity. In the case of ERα, this switch may be operated by agonist versus antagonist binding. In order to define the roles of p160 proteins and PBP in the assembly and activities of the ER transcription complex, we utilized chimeric coactivator/corepressor proteins in which the three NR boxes in GRIP1 and the two NR boxes in PBP had been replaced by CoRNR boxes (Figure 5A). These CoRNR box *sub*stituted cofactors termed subGRIP and subPBP are recruited to the unliganded and helix 12 deleted form of RXR in vitro while the wild-type proteins are not, confirming their reversed specificity (Figure 5B).

The substituted cofactors were expressed in MCF-7 cells either separately or together, and promoter occupancy after treatment with E2 or TAM was assessed by ChIP. When wild-type GRIP1 was expressed in MCF-7 cells, as expected, only ERα was recruited to the CATD promoter in response to TAM (Figure 5C, upper left panel). As control, when these cells were treated with E2, the recruitment of ERα, GRIP1, CBP, and PBP was observed (Figure 5C, lower left panel). In marked contrast, after introduction of subGRIP1 into MCF-7 cells, TAM was able to induce the recruitment of not only ERα, but also GRIP1 and CBP (Figure 5C, upper left panel). Remarkably, subGRIP1 transfection led to histone acetylation in response to TAM, suggesting that subGRIP1 was sufficient to induce an activated chromatin template on the CATD promoter. Transcriptional activation was confirmed by cotransfection of subGRIP1 with an estrogen-responsive luciferase reporter (Figure 5C, right panel). Importantly PBP was not recruited by TAM-ERα even in the presence of subGRIP1, suggesting that PBP recruitment is not a necessary component of an active ERα transcription complex.

To further assess the role of PBP in ERα-mediated gene activation, MCF-7 cells were transfected with either wild-type PBP or CoRNR box-substituted PBP, subPBP, and treated with TAM. As expected transfection of wild-type PBP did not facilitate the recruitment of PBP, GRIP1, or CBP in response to TAM. Surprisingly however, transfection of subPBP gave the same results suggesting that substitution of the CoRNR box for the NR box in PBP was not sufficient to promote its recruitment by TAM-bound ERα nor to allow gene activation (Figure 5C, top panels), even though in control experiments PBP recruitment and gene activation were observed in both PBP and subPBP-transfected cells treated with E2 (Figure 5C, lower panels). Interestingly, when both subGRIP1 and subPBP were introduced into cells, PBP was recruited to the ERα complex by TAM and the addition of subPBP increased the level of activation compared to that seen with subGRIP1 alone (Figure 5C, top panels). These differences were not due to differences in protein expression as measured by Western blotting (Figure 5D). Taken together these results suggest that while substitution of the CoRNR box for the NR box is sufficient for recruitment of p160 coactivators and ERα-mediated gene activation, the recruitment of PBP to the ERα complex requires both an interaction with ERα and the presence of a p160 factor.

To determine whether the sufficiency of p160 action

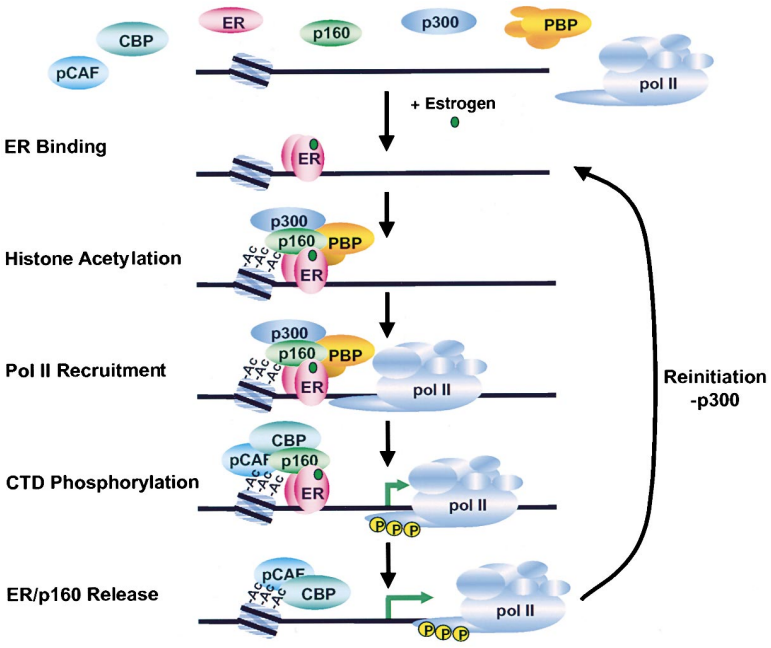

Figure 7. Cyclic Model of ER Transcription Complex Assembly

The sequential formation of complexes leading to the activation of gene expression by agonists such as estradiol. See text for details.

for gene activation extends to a physiologically relevant response in breast cancer cells, we examined the effects of TAM on cell cycle progression of MCF-7 cells expressing subGRIP1. Estrogen is normally required for G_1/S transition of MCF-7 cells and estrogen deprivation leads to a significant G_1 arrest. We cotransfected estrogen-deprived MCF-7 cells with GRIP1 or subGRIP1 together with a green fluorescent protein (GFP) expression construct. Cells then were treated with E2 or TAM and the cell cycle profile of the GFP-expressing population was determined by flow cytometry (Figure 6). In E2-deprived MCF-7 cells expressing either wild-type GRIP1 or subGRIP1, ~85% of the GFP-expressing cells were arrested in the G_0/G_1 phase of the cell cycle. E2 addition for 16 hr was able to release ~15% of the GFP-expressing cells into cell cycle with the G_0/G_1 fraction changing from ~85% to ~70%. Treatment of wild-type GRIP1-expressing cells with TAM for 16 hr had no effect on the cell cycle profile with ~85% of cells remaining in G_0/G_1. In marked contrast, in cells expressing subGRIP1, TAM treatment was able to effect the release of ~11% of the cells into the cell cycle. These results indicate that the recruitment by ERα of a p160 coactivator is sufficient to exert the cell proliferating properties of estrogen in breast cancer.

Discussion

Dynamics of ER Transcription Complex Assembly

Genetic and biochemical studies over the past two decades have revealed that the process of gene activation in eukaryotic cells is extremely complicated. Chromatin immunoprecipitation (ChIP) is a powerful technique that offers the advantage of being able to detect endogenous transcription factors bound in vivo to promoters under different physiologic conditions. In addition to direct promoter binding factors, the presence of proteins that are not bound directly to DNA and that depend on other proteins for promoter binding can also be determined using ChIP. Using ChIP, we found that p160 coactivators, CBP, p300, pCAF, and PBP are recruited in a specific order to the ER transcription complex after estrogen stimulation in MCF-7 breast cancer cells.

Interestingly, the ER transcription complex appears to repeatedly cycle onto and off of target promoters in the presence of continuous stimulation by estrogen. The regular cycling of the ERα transcription complex may represent a mechanism that favors continuous sampling of the external milieu. Cycling may be regulated in part by covalent modification of coregulators (Chen et al., 1999; Font De Mora and Brown, 2000; Rowan et al., 2000). In addition our inhibitor studies suggest that the cycling of the ERα complex off the promoter depends on the phosphorylation of the C-terminal domain of the large subunit of RNA pol II. Factors that alter the phosphorylation of RNA pol II might affect the responsiveness of ERα-dependent promoters by interfering with the cycling of ERα complex.

We confirmed that TAM induces the recruitment of ERα to responsive promoters. We did not observe recruitment of coactivators in response to TAM in MCF-7 cells, where TAM is a full antagonist. Consistent with in vitro studies (Jackson et al., 1997; Smith et al., 1997; Lavinsky et al., 1998), TAM-bound ERα did recruit nuclear receptor corepressors N-CoR and SMRT to the promoters as well. Preliminary data suggests that TAM-ERα actively represses transcription of genes to which it recruits these corepressors (data not shown).

Functional Specificity and Sufficiency of Coactivators

Although several lines of evidence point to functional differences between CBP and p300, (reviewed by Glass and Rosenfeld, 2000), evidence for functional differences among CBP, p300, and pCAF in ERα-mediated transcription has been lacking. Our experiments show

that p300, CBP and pCAF are all involved in ERα-mediated gene transcription and that in the first cycle of transcription initiation these three proteins are sequentially recruited to an ERα transcription complex, with p300 first, followed by CBP and pCAF. These findings agree well with a previous observation that p300 interacts specifically with the nonphosphorylated, initiation-competent form of RNA polymerase II, whereas pCAF interacts with the elongation-competent, phosphorylated form (Cho et al., 1998). Our observation that p300 is not recruited in subsequent cycles is consistent with in vitro transcription data suggesting that while p300 plays a role in transcription initiation by ERα, it does not participate in reinitiation (Kraus and Kadonaga, 1998). These results may also indicate that histone acetylation and chromatin remodeling could be a step-wise process in which each of these three cofactors exerts a distinct and nonredundant role and each of these three HAT proteins exhibits a different substrate specificity, as suggested by in vitro studies (Schiltz et al., 1999).

The PBP/DRIP/TRAP complex has been proposed to represent a distinct complex from the ERα-p160 complex and to act at a later stage in gene activation, after histone acetylation by the p160 complex makes chromatin more accessible. Our data show that PBP is recruited to ERα responsive promoters rapidly after estrogen stimulation, at about the same time as p160 factors and p300. In addition, our ChIP Re-IP results argue that the p160 complex acts in combination with the PBP complex on the same ERα responsive promoter rather than the two complexes acting independently from each other on different promoters.

The reversed pharmacology of GRIP1 and PBP with CoRNR box substitutions allowed us to determine which of these coactivators are sufficient for binding and activation of an ERα complex. Our results indicate that recruitment of a p160 coactivator is sufficient to induce assembly of an ERα complex capable of gene activation without the recruitment of PBP. In contrast recruitment of PBP requires both an interaction with ERα and the presence of the p160 coactivator in the complex. The CoRNR box–substituted p160 factor is not only sufficient to promote the assembly of an active transcription complex, but was sufficient to reverse the effects of TAM and promote cell cycle progression in MCF-7 cells. This supports a pivotal role for p160 coactivators in estrogen signaling in breast cancer as suggested by the amplification of AIB1 in a subset of ER-positive breast cancers.

Based on our findings, we propose a dynamic model for the cyclic assembly of ERα transcription complexes (Figure 7). Rapidly, upon the addition of an agonist such as E2, liganded-ERα binds DNA. This is almost immediately followed by the recruitment of both a HAT-containing p160-p300 complex and the PBP complex. The p300 HAT complex modifies local chromatin structure through histone acetylation to facilitate RNA pol II recruitment. p300 acts in the initial cycle of transcription initiation, but not in subsequent cycles, perhaps suggesting that histone acetylation by p300 is long lived. Concurrent with the onset of transcription, the pol II C-terminal domain is phosphorylated and CBP replaces p300 in the complex bringing in pCAF. Subsequently, CBP acetylates p160 and leads to the release of p160 along with ERα. Finally, CBP and pCAF disassemble and the cycle is repeated. It is likely that cell-specific factors contribute to selective modulation of ER dynamics and cofactor sufficiency in important target tissues other than the breast, including the uterus, skeleton, brain, and cardiovascular system.

Experimental Procedures

Materials and Reagents

Antibodies used: αERα: Ab-1, Ab-3, and Ab-10 (NeoMarkers, Fremont, CA); αAIB1 (affinity purified rabbit polyclonal serum), αCBP (AC26) and αp300 (RW128) (D. M. Livingston, Dana-Farber Cancer Institute, Boston, MA); αpCAF (Y. Nakatani, Dana-Farber Cancer Institute, Boston, MA); αRNA polymerase II: 8WG16 (J. B. Parvin, Brigham and Women's Hospital, Boston, MA) and H5 (Berkeley Antibody Company, Richmond, CA); αAcetylated histone (Upstate Biotechnology, Inc., Lake Placid, NY); αPBP (Atkins et al., 1999); αSMRT (Guenther et al., 2000); αN-CoR (Huang et al., 2000); αVDR (Affinity BioReagents, Inc., Golden, CO).

ChIP

Cells were grown to 95% confluence in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days. Following the addition of hormone for various times, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. Cells then were rinsed with ice-cold PBS twice and collected into 100 mM Tris-HCl (pH 9.4), 10 mM DTT and incubated for 15 min at 30°C and centrifuged for 5 min at 2000 g. Cells were washed sequentially with 1 ml of ice-cold PBS, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5). Cells were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1× protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated three times for 10 s each at the maximum setting (Fisher Sonic Dismembrator, Model 300) followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immunoclearing with 2 μg sheared salmon sperm DNA, 20 μl preimmune serum and protein A-sepharose (45 μl of 50% slurry in 10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 2 hr at 4°C. Immunoprecipitation was performed for 6 hr or overnight at 4°C with specific antibodies. After immunoprecipitation, 45 μl protein A-Sepharose and 2 μg of salmon sperm DNA were added and the incubation was continued for another 1 hr. Precipitates were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed three times with TE buffer and extracted three times with 1% SDS, 0.1 M $NaHCO_3$. Eluates were pooled and heated at 65°C for at least 6 hr to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA). For PCR, 1 μl from a 50 μl DNA extraction and 21–25 cycles of amplification were used.

Nuclear Run-on

MCF-7 cells were grown in estrogen-depleted media for 3 days and treated with 100 nM of E2 for various times. Cell nuclei were isolated with NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% [v/v] NP-40) and stored in liquid nitrogen in glycerol storage buffer (50 mM Tris.HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). Nuclear run-on transcription was performed in the presence of 5 μl of 10 mCi/ml [α-^{32}P]UTP. After treatment of the reaction with RNase-free DNase I and proteinase K, RNA was extracted with phenol/chloroform/isoamyl alcohol and hybridized to c-Myc and pS2 cDNAs, cathepsin D gene exon 1 DNA and β-actin oligo probe (Oncogene Research Products, Cambridge, MA) immobilized on a nylon membrane.

ChIP Re-IP and Real-time PCR

Complexes were eluted from the primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by reimmunoprecipitation with the second antibodies. ChIP Re-IPs of supernatants were done essentially as were the primary IPs. For real-time PCR detection of cathepsin D promoter, the probe and primers were: 6FAM-CCAAGGTTAAATT CAAAGTCCCCAGC-TAMRA (probe); TCCAGACATCCTCTCTGGAA (forward primer); GGAGCGGAGGGTCCATTC (reverse primer). The ABI PRISM 7700 Sequence Detector and TaqMan 1000 Rxn PCR Core Reagents (Perkin Elmer, Branchburg, NJ) and sixty cycles of amplification were used.

Construction of CoRNR Box–Containing GRIP1 and PBP

CoRNR box–containing GRIP1 (subGRIP1) and PBP (subPBP) were constructed by standard molecular techniques. Each of the three NR boxes in GRIP1 was changed to the CoRNR box sequence -LEDIIRKALMGSFD- and both PBP NR boxes were changed to the chimeric CoRNR box sequence -HRLITLADHIEDIIRKALMG- .

Flow Cytometry

MCF-7 cells were grown in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped fetal bovine serum for 24 hr and were cotransfected with pcDNA3-GFP and pCMX-GRIP1 or pcDNA3-subGRIP1. Forty-eight hours after the transfection, cells were treated with 100 nM E2 or 5 μM TAM for another 16 hr. Cells were then collected and stained with propidium iodide using standard methods. Cell cycle data were collected with FACScan (Becton Dickinson Immunocytochemistry System) and analyzed with ModFit LT (Verity Software House, Inc., Topsham, ME).

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