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Formation of the Androgen Receptor Transcription Complex

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Summary

Androgen receptor (AR) is required for sexual differentiation and is implicated in the development of prostate cancer. Here we describe distinct functions for cofactor proteins and gene regulatory elements in the assembly of AR-mediated transcription complexes. The formation of an activation complex involves AR, coactivators, and RNA polymerase II recruitment to both the enhancer and promoter, whereas the formation of a repression complex involves factors bound only at the promoter and not the enhancer. These results suggest a model for the functional coordination between the promoter and enhancer in which communication between these elements is established through shared coactivators in the AR transcription complex.

Introduction

The androgen receptor (AR), a member of the nuclear receptor superfamily functioning as a ligand-dependent transcription factor, mediates male sexual differentiation in utero, sperm production at puberty, prostate development in the adult, and primary prostatic cancer growth in prostate cancer patients (Brinkmann and Trapman, 2000). The genes activated by AR often contain enhancer and promoter elements in their regulatory regions. This is the case for the gene encoding for the prostate-specific antigen (PSA), the best-characterized androgen-responsive gene in the prostate gland. Biochemical and genetic studies revealed that both the enhancer and the promoter in the PSA gene display androgen responsiveness but a maximal activity requires the presence of both (Cleutjens et al., 1996, 1997a, 1997b; Huang et al., 1999; Pang et al., 1995, 1997; Reid et al., 2000; Schuur et al., 1996; Sun et al., 1997; Zhang et al., 1997). The proximal promoter has been localized to a ~630 bp fragment containing a core TATA box and two putative androgen-responsive elements (AREs), ARE I, and ARE II (Cleutjens et al., 1996; Pang et al., 1995). The AR activates transcription synergistically through these AREs. The enhancer element, centered at approximately 4.2 kb, is located within a 6 kb region (Cleutjens et al., 1997b) and harbors another putative ARE, termed ARE III. How the enhancer and the promoter, almost 4 kb apart, are able to coordinate the assembly of an AR transcription complex is currently unknown.

Like other members of the nuclear receptor superfamily, the AR contains distinct structural and functional domains consisting of a central DNA binding domain (DBD), a C-terminal ligand-binding domain (LBD), and two potential transcription activation domains (AF-1 and AF-2). AF-1 is located in the N-terminal region of the receptor and has been shown to be a ligand-independent activation domain. A ligand-dependent AF-2 domain, which colocalizes with the highly conserved LBD, is predicted from the sequence similarity between AR and other nuclear receptors (Beato et al., 1996; Brinkmann et al., 1999; Li et al., 2000). However, an AF-2 for AR is less well defined than for many of the other steroid receptors such as estrogen receptor (ER) (Jenster et al., 1991, 1992; Matias et al., 2000; Rundlett et al., 1990; Simental et al., 1991; Zhou et al., 1994). Nuclear receptors modulate the rate of transcriptional initiation through interactions with the basal transcription machinery and through alterations in the state of chromatin organization at the promoter of target genes (Beato et al., 1996; Glass and Rosenfeld, 2000; Lemon and Freedman, 1999). These processes are dependent on the integrity of AF-2 and involve the recruitment of a variety of cofactors such as histone acetyltransferase (HAT) activity-containing proteins or histone acetylases (Durand et al., 1994; Jenster et al., 1997; Korzus et al., 1998; Lemon and Freedman, 1999; Wolffe et al., 1997; Xu et al., 1999).

Histone acetylation has been correlated with transcriptionally active genes and the function of a transcriptional coactivator. The rates of gene transcription roughly correlate with the degree of histone acetylation, with hyperacetylated regions of the genome being more actively transcribed than hypoacetylated regions (Pazin and Kadonaga, 1997). Thus, the specific recruitment of a complex with HAT activity to a promoter may play a critical role in overcoming repressive effects of chromatin structure on transcription (Pazin and Kadonaga, 1997; Struhl, 1998; Wade and Wolffe, 1999). The coactivator proteins that possess HAT activity and are implicated in nuclear receptor-mediated gene transcription include the p160 family of coactivators, including SRC-1, TIF2/GRIP1-1, and ACTR/AIB1/RAC3/pCIP (Anzick et al., 1997; Chen et al., 1997; Hong et al., 1996; Li et al., 1997; Onate et al., 1995; Takeshita et al., 1997), CBP, p300 (Chakravarti et al., 1996; Hanstein et al., 1996), and pCAF (Blanco et al., 1998). The lack of a robust AF-2 function in AR has made the demonstration of the participation of these cofactor proteins in AR-mediated gene transcription in vivo somewhat problematic.

Conversely, the state of gene repression is correlated with histone deacetylation by corepressors and their associated histone deacetylases (HDACs) (Hu and Lazar, 2000; Nagy et al., 1997; Ng and Bird, 2000; Perissi et al., 1999; Wen et al., 2000). Unlike retinoic acid receptor (RAR) and thyroid hormone receptor (TR) that are capable of gene repression by interacting with corepressors NCoR and SMRT and recruiting HDAC activities, steroid

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receptors, including AR and ER, do not repress gene transcription in the absence of hormones (Hu and Lazar, 2000). Rather, it has been suggested that, at least for ER, the switch from gene activation to gene repression is accomplished through antagonist binding (Hu and Lazar, 2000). As evidence, antagonist-bound ERs have been demonstrated to be able to interact with corepressors in vitro (Jackson et al., 1997; Smith et al., 1997) and to be associated with corepressors and HDACs in vivo (Shang et al., 2000). The structural and functional similarity between ER and AR and the pharmacological similarity between antiestrogens and antiandrogens suggests that a similar corepression complex may be recruited by antagonist-bound AR.

Here we describe the role played by HAT-containing coactivator complexes in the dynamic assembly of an agonist-activated AR transcription complex. In addition we show that, as predicted, corepressor-HDAC complexes are recruited by antagonist-bound AR. The formation of an active coactivator complex involves the recruitment of AR to both the enhancer and promoter, followed by the recruitment of coactivators including histone acetylases and RNA polymerase II. In contrast, the formation of an AR repression complex only involves factors bound at the promoter and not the enhancer. These results suggest a model of the AR transcription complex in which coactivators serve as a bridge that functionally links the promoter and enhancer.

Results

Histone Acetylation in AR-Mediated Gene Transcription

The best-characterized androgen-responsive gene in LNCaP prostate cancer cells is the gene encoding prostate-specific antigen (PSA), which is highly expressed in the luminal epithelial cells of the normal prostate and has been used as a prostate-specific tumor marker (Brawer, 2000). In order to establish the importance of histone acetylation, and thus, of the recruitment of histone acetylases during AR-mediated gene transcription, we examined the effects of alterations in the state of histone acetylation on AR-mediated PSA gene transcription. For these studies, the 6 kb androgen-responsive PSA gene regulatory region (Cleutjens et al., 1997a) was fused to a luciferase reporter. The state of histone acetylation was modulated using the HDAC inhibitor, trichostatin A (TSA) (Yoshida et al., 1990) or by overexpression of HDAC1 in LNCaP cells. Dihydrotestosterone (DHT)-induced luciferase activity was enhanced by TSA (Figure 1A), whereas overexpression of HDAC1 counteracted the DHT-stimulated reporter gene activity. The effect of HDAC1-overexpression could be reversed by addition of TSA. In addition, endogenous PSA expression was measured in LNCaP cells after treatment with DHT alone or with DHT plus TSA. As expected, PSA expression was strongly stimulated by DHT treatment (Figure 1B). Consistent with the reporter assays, this stimulation was further enhanced in the presence of the HDAC inhibitor TSA. Collectively, these data strongly indicate a correlation between the state of histone acetylation and PSA gene activation and suggest that the recruitment of histone acetylases is critical in the assembly of the AR transcription complex.

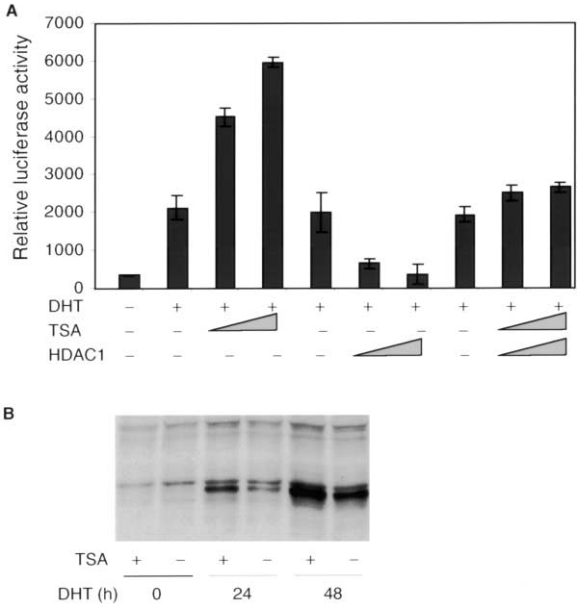

Figure 1. Histone Acetylation in AR-Mediated Gene Transcription

(A) The effects of trichostatin A (TSA) and HDAC1 on DHT-stimulated gene transcription. LNCaP cells were transfected with 50 ng of a PSA promoter-driven luciferase along with 100 ng or 500 ng of an HDAC1 expression construct. After 48 hr of transfection, cells were treated with 10 nM of DHT or with 0, 50, or 200 nM of TSA for 16 hr, and the luciferase activity was measured. Transfection efficiency was normalized using a β-galactosidase expression construct, and the results represent at least three independent experiments.

(B) Western blot analysis of PSA expression in LNCaP cells under treatment with DHT or DHT plus TSA. LNCaP cells were treated with 10 nM DHT or 10 nM DHT plus 100 nM TSA for different times. Total proteins were extracted from cells, and Western blotting was performed using specific antibodies against PSA.

AR Binds Both the Enhancer and the Promoter of the PSA Gene

Three AREs have been identified in the PSA gene: ARE I and ARE II are in the ~630 bp promoter region, whereas ARE III resides in the enhancer region located ~4 kb upstream of the PSA transcription start site (Figure 2A) (Cleutjens et al., 1997b). To determine how the enhancer and the promoter coordinate AR-mediated PSA gene transcription, chromatin immunoprecipitation (ChIP) assays were performed to detect the occupancy of AR in these two elements after treatment with DHT. LNCaP cells were grown in phenol red-free RPMI Medium 1640 supplemented with 5% charcoal-dextran-stripped fetal bovine serum for at least 3 days followed by treatment with 10 nM DHT. Soluble chromatin was prepared after formaldehyde treatment of the cell cultures. Specific antibodies against AR were used to immunoprecipitate AR-bound genomic DNA fragments. The genomic DNAs were analyzed by PCR using specific pairs of primers spanning the AREs (Figure 2A). AR recruitment was detected to both ARE I (with primer pair A/B) and ARE II (with primer pair C/D) in LNCaP cells after treatment with DHT (Figure 2B). In addition, induced occupancy of AR in response to DHT treatment was also observed at the enhancer region with primer pair G/H, suggesting that ARE III in the enhancer is also bound by AR. As the

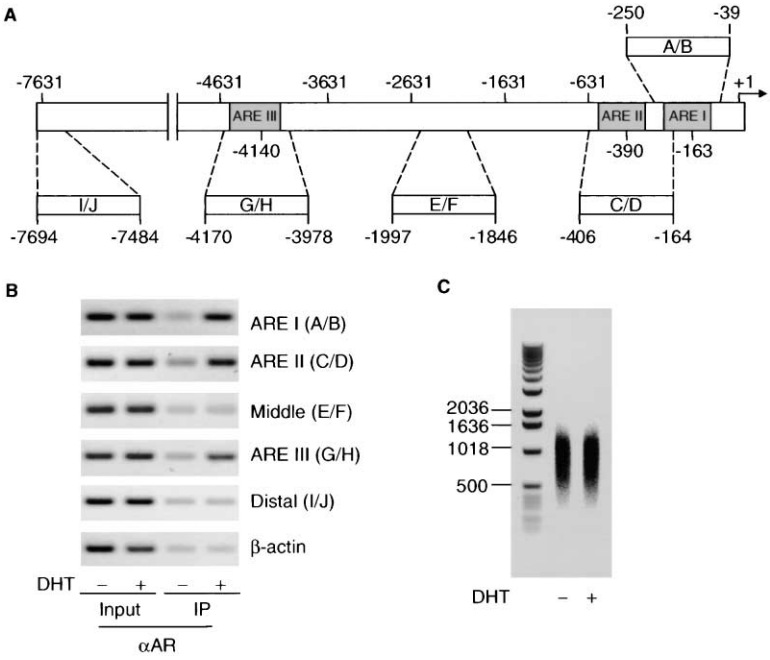

Figure 2. AR Recruitment in the Enhancer and the Promoter of the PSA Gene in Response to DHT Treatment

(A) Schematic diagram of the PSA gene regulatory region. Solid boxes depict putative AREs, and A/B, C/D, E/F, G/H, and I/J are primer pairs used for amplifying corresponding DNA fragments. The numbers are the positions upstream to the PSA gene transcription start site.

(B) ChIP assays of AR occupancy on the PSA gene regulatory region. LNCaP cells were treated with 10 nM DHT. Soluble chromatin was prepared from formaldehyde-cross-linked and sonicated cell cultures. Specific antibodies against AR were used to immunoprecipitate protein-bound DNA fragments. These fragments were amplified by PCR using primers described in (A).

(C) The size of the genomic DNA fragments from solubilized chromatin as described in (B).

average length of the genomic DNA fragments produced in these experiments is ~750 bp (Figure 2C), we cannot distinguish with certainty whether either ARE I or ARE II or both are bound by AR. Nevertheless, the binding of AR both to the promoter and to the enhancer regions is specific as there was no DHT-induced AR binding to the region between the promoter and enhancer detected with primer pair E/F nor to a position distal to the enhancer located ~7 kb region upstream of the PSA transcription start site (primer pair I/J). In addition, no AR binding was detected to the promoter of the nonandrogen-responsive gene, β-actin (Figure 2B).

Coordination of the Enhancer and Promoter in the Assembly of AR Coactivator and Corepressor Complexes

The "Yin-Yang" in transcription regulation by nuclear receptors is reflected in gene activation-repression. Nuclear receptors activate gene transcription through the recruitment of coactivators that modify chromatin structure and interact with the basal transcriptional machinery. Histone acetylases and histone acetylation are the best-characterized coactivators and chromatin-remodeling activities in nuclear receptor-mediated gene transcription. These proteins include AIB1, GRIP1, and SRC-1, CBP, p300 and pCAF. Nuclear receptors such as RAR and TR that repress transcription in the absence of ligands are able to recruit a corepressor complex. This complex contains nuclear receptor corepressors NCoR and SMRT and their associated HDACs. Unliganded steroid receptors such as ER and AR have not been associated with a corepressor complex. However, antagonist-bound ER has been demonstrated to be able to interact with corepressors (Jackson et al., 1997; Smith et al., 1997) in vitro and recruit a corepressor complex to target promoters in vivo (Shang et al., 2000).

In order to investigate the roles of coactivators and corepressors in AR function, we first confirmed the agonistic activity of DHT and antagonistic activity of bicalutamide (ICI 176344 or Casodex) in LNCaP cells. Cells were grown in phenol red-free RPMI Medium 1640 supplemented with 5% charcoal-dextran-stripped fetal bovine serum for 3 days and treated with DHT or DHT plus bicalutamide for various times. As expected, DHT treatment significantly enhanced the expression of PSA, and the addition of bicalutamide totally abolished DHT-induced PSA expression (Figure 3A).

To determine the participation of coactivators or corepressors in AR agonistic activity or antagonistic activity, and to investigate the roles of the promoter and enhancer in coordinating such activities, we measured the recruitment of AR, the coactivators GRIP1 and CBP, the corepressors NCoR and SMRT, and HDAC1 and HDAC2 in both the enhancer and the promoter by ChIP assays (Figure 3B). AR recruitment was observed in both the promoter region (right panel) and the enhancer region (left panel) after treatment with DHT (D) but only to the promoter region after bicalutamide treatment (B). DHT induced the recruitment of coactivators GRIP1 and CBP to both the enhancer and the promoter, whereas the recruitment of these proteins was not detected after treatment with bicalutamide. However, bicalutamide-bound AR was able to recruit corepressors NCoR and SMRT, and HDAC1 and HDAC2. Intriguingly, similar to the recruitment of AR, the recruitment of these cofactors was only detected in the promoter region (Figure 3B, right panel) but not in the enhancer region (Figure 3B, left panel). The steady-state expression level of AR and the coregulators was not changed by treatment with DHT or bicalutamide (data not shown). These data indicate that coactivator complex formation initiated by agonist-bound AR involves coordination between both the promoter and enhancer, whereas corepressor complex formation initiated by antagonist-bound AR only involves the promoter.

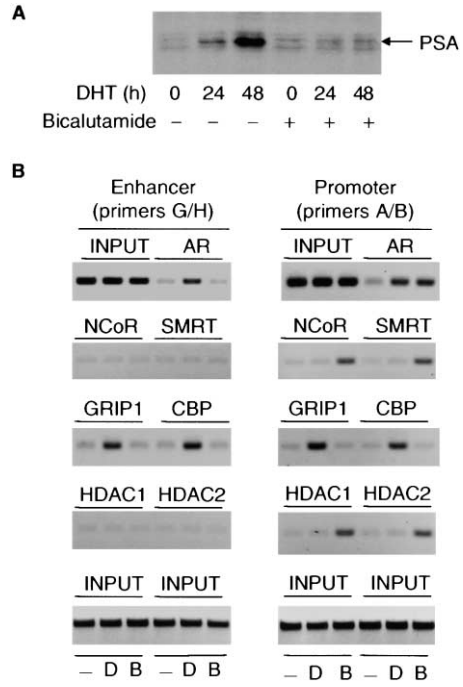

Figure 3. Distinct Roles of the Enhancer and Promoter in the Assembly of AR Coactivator and Corepressor Complexes

(A) The DHT induction of PSA expression is blocked by bicalutamide in LNCaP cells. LNCaP cells were treated with 10 nM DHT or 10 nM DHT and 5 μM bicalutamide for different times. Total proteins were extracted and Western blotting was performed to detect PSA expression.

(B) ChIP assays reveal differential recruitment of coactivators and corepressors to the PSA promoter and enhancer. LNCaP cells were treated with 10 nM DHT (D), 5 μM bicalutamide (B), or vehicle alone (–) for 1 hr. Soluble chromatin was prepared and immunoprecipitated with antibodies against AR, GRIP1, CBP, NCoR, SMRT, HDAC1, and HDAC2. Final DNA fragments were PCR-amplified using the primer pair A/B to detect promoter binding (right panel) or primer pair G/H to detect enhancer binding (left panel).

The Essential Role of the p160-CBP/p300 Interface in the Assembly of the AR Coactivator Complex

Next, we investigated how AR might interact with coactivators and how coactivators might interact with each other in the assembly of the AR transcription complex. In contrast to p160 proteins, ligand-dependent interaction between CBP and nuclear receptors is weak, whereas the interaction between CBP and p160 proteins and between fragments of these proteins is much stronger in vitro (Sheppard et al., 2001). In addition, it has been demonstrated that deletion of the N-terminal nuclear receptor binding region does not disrupt the ability of CBP or p300 to stimulate nuclear receptor activity (Kraus et al., 1999; McInerney et al., 1998; Westin et al., 1998), whereas deletion of the p160 binding region markedly abrogates p300 coactivation potential (Kraus et al., 1999; Li et al., 2000). Collectively, these data suggest that the recruitment of CBP and p300 occurs through an interaction of CBP and/or p300 with the p160 protein rather than a direct interaction between nuclear receptors and CBP or p300. We thus sought to determine whether the interface between CBP or p300 and a p160 protein is essential in the formation of the AR transcription complex. The sufficiency of a p160 protein in mediating the recruitment of CBP and gene transactivation by ER was established by our recent report utilizing a GRIP1 mutant protein with reversed pharmacology (Shang et al., 2000). This mutant protein, termed subGRIP1, contains CoRNR boxes (corepressor binding motifs) substituted for NR boxes (coactivator binding motifs). It is capable of binding helix 12-deleted RXR and tamoxifen-bound ER and is sufficient to coactivate estrogen target gene transcription in the presence of the ER antagonist tamoxifen (Shang et al., 2000).

CBP and p300 interact with p160 proteins through activation domain 1 (AD1) located near the C terminus of the p160 proteins. Another activation domain, AD2, located at the extreme C terminus, has been shown to bind methyltransferases CARM1 and/or PRMT1 in ER-mediated transcription (Koh et al., 2001). In order to determine the essential role of the p160-CBP/p300 interface in the assembly of the AR transcription complex, we generated mutants of subGRIP1 with either AD1 or AD2 deleted, termed subGRIP1ΔAD1 and subGRIP1-ΔAD2, respectively (Figure 4A). LNCaP cells were transfected with wild-type GRIP1, subGRIP1, subGRIP1ΔAD1, or subGRIP1ΔAD2 along with the PSA 6 kb androgen-responsive regulatory region-luciferase construct. Cells were then treated with DHT or bicalutamide, and luciferase activity was measured. Reporter activity was modestly enhanced by GRIP1 cotransfection only in cells treated with DHT and not with bicalutamide (Figure 4B). In contrast, subGRIP1 was able to augment the level of reporter activity only in cells treated with the antagonist and not with agonist consistent with its reversed pharmacology. Furthermore, while subGRIP1ΔAD2 had little effect on bicalutamide-stimulated luciferase activity, the deletion of AD1 in subGRIP1ΔAD1 abolished bicalutamide-stimulated luciferase activity. Parallel experiments indicated that the lack of subGRIP1ΔAD1 coactivator activity was not due to the inability of this mutant to be recruited to DNA by bicalutamide-bound AR as it was detected by ChIP (Figure 4C). In addition, wild-type GRIP1 and the GRIP1 mutants were all expressed in these experiments to similar levels (Figure 4D). Rather, the inability of subGRIP1ΔAD1 to mediate AR-activated luciferase activity was due to the failure of this mutant to recruit CBP and to induce histone acetylation (Figure 4C). These results strongly indicate that p160-CBP/p300 interface is essential in the formation of an active AR transcription complex.

The reporter activation (Figure 4B) and histone acetylation (Figure 4C) observed with subGRIP1 and subGRIP1ΔAD2 transfections in cells treated with bicalutamide suggest that the recruitment of a p160 protein is sufficient to support AR-mediated gene activation. In order to determine whether the sufficiency of p160 action for gene activation can be extended to a physiologically relevant response in prostate cancer cells, we examined the effects of bicalutamide on cell cycle progression of LNCaP cells expressing subGRIP1. Androgen stimulates the G_1/S transition in LNCaP cells, and androgen deprivation leads to a significant G_1 arrest. We cotransfected androgen-deprived LNCaP cells with GRIP1, subGRIP1, subGRIP1ΔAD1, or subGRIP1ΔAD2 together with a green fluorescent protein (GFP) expression construct. Cells were then treated with DHT or

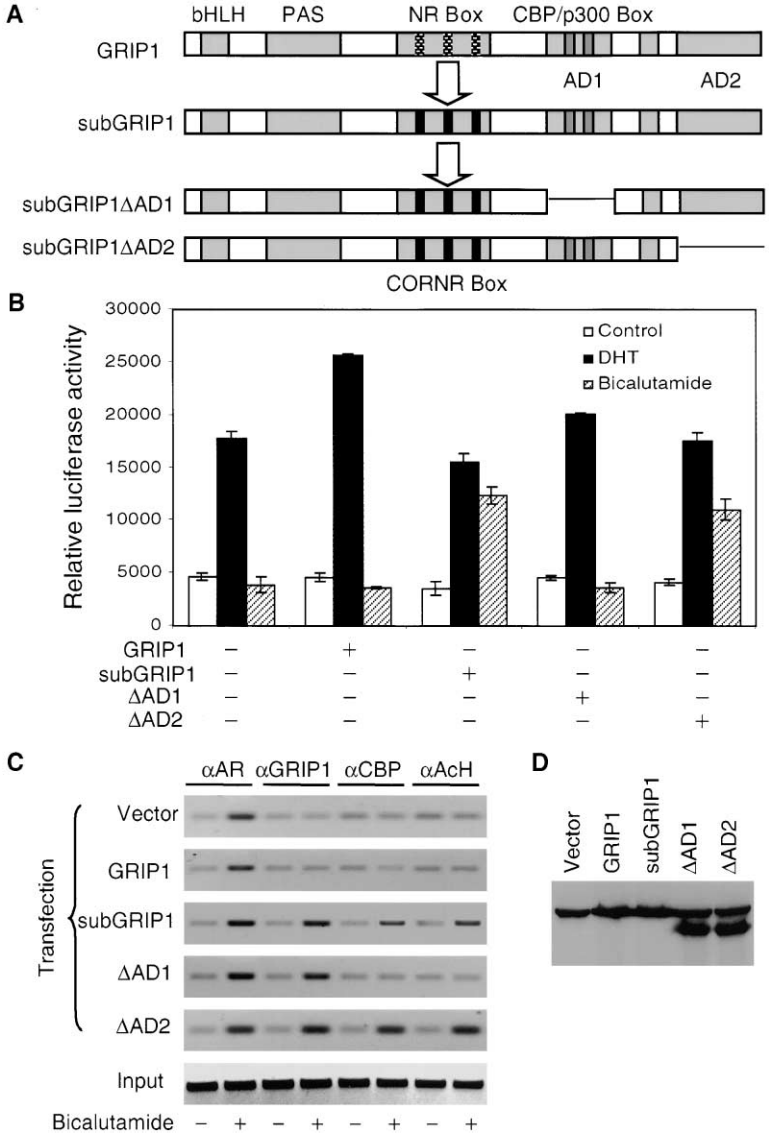

Figure 4. An Essential Role of the p160-CBP/p300 Interface in the Assembly of AR Coactivator Complex

(A) Diagrams of the functional domains of GRIP1, subGRIP1, subGRIP1ΔAD1, and subGRIP1ΔAD2. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; NR box, Nuclear receptor binding motif; CoRNR box, corepressor nuclear receptor binding motif; AD, activation domain.

(B) Deletion of the AD1 in subGRIP1 diminishes bicalutamide-stimulated gene transcription. LNCaP cells were transfected with 500 ng of GRIP1, subGRIP1, subGRIP1ΔAD1 (ΔAD1), or subGRIP1ΔAD2 (ΔAD2). Forty-eight hours after transfection, cells were treated with 10 nM DHT or 5 μM bicalutamide. Luciferase activity was measured and normalized with a β-galactosidase expression construct; the results represent at least three independent experiments.

(C) Diminished AR activity in subGRIP1ΔAD1 transfection is not due to the inability of subGRIP1ΔAD1 to bind to bicalutamide-bound AR on PSA gene promoter. ChIP experiments were performed under the same experimental conditions described in (B) to measure the recruitment of GRIP1 or GRIP1 mutants, CBP, and the acetylation of histone (AcH).

(D) Diminished AR activity in subGRIP1ΔAD1 transfection is not due to the failure of subGRIP1ΔAD1 expression. Western blotting was performed under the same experimental conditions described in (B) to measure the expression of GRIP1 and GRIP1 mutants using GRIP1-specific antibodies.

bicalutamide, and the cell cycle profile of the GFP-expressing population was determined by flow cytometry (Figure 5). In androgen-deprived LNCaP cells expressing either wild-type GRIP1 or subGRIP1, ~80% of the GFP-expressing cells were arrested in the G_0/G_1 phase of the cell cycle. DHT addition for 16 hr was able

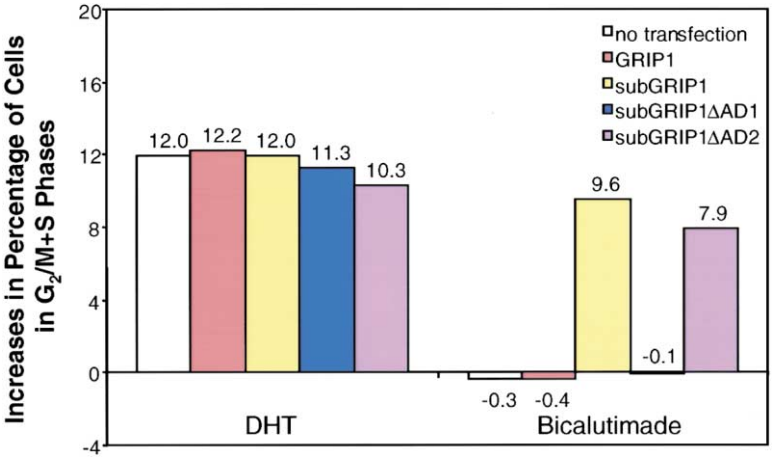

Figure 5. Induction of Cell Cycle Progression in LNCaP Cells Expressing subGRIP1

LNCaP cells were transfected with GRIP1, subGRIP1, subGRIP1ΔAD1 (ΔAD1), or subGRIP1ΔAD2 (ΔAD2) along with a GFP construct. Forty-eight hours after transfection, cells were treated with 10 nM DHT or 5 μM bicalutamide or vehicle alone for another 16 hr. Cells were then collected and analyzed by flow cytometry. The numbers represent the percentage of GFP-positive cells that are in the $S/G_2/M$ phases of the cell cycle after 16 hr treatment with DHT or bicalutamide.

to release ~12% of the GFP-expressing cells into cell cycle, with the G_0/G_1 fraction changing from ~80% to ~68%. Treatment of wild-type GRIP1-expressing cells with bicalutamide for 16 hr had no effect on the cell cycle profile, with ~80% of cells remaining in G_0/G_1. In marked contrast, in cells expressing subGRIP1, bicalutamide treatment was able to effect the release of ~10% of the cells into the cell cycle. This effect was totally abolished when the p160-CBP/p300 interface was eliminated by transfection of subGRIP1ΔAD1, whereas AD2 deletion had little effect. These results indicate that the recruitment by AR of a p160 coactivator is sufficient to initiate the formation of a competent AR transcription complex and exert the cell proliferating properties of androgen in prostate cancer. In addition the requirement for an intact AD1 domain supports the importance of the recruitment of p300 and/or CBP by p160 proteins.

A Model for the Coordination between the Enhancer and Promoter in the Assembly of the AR Transcription Complex

Our observation that AR occupies both the promoter and the enhancer of PSA gene in response to agonist binding (Figure 2) suggests a possible explanation as to why both the promoter and the enhancer are needed for maximal AR activity (Cleutjens et al., 1997b). Given that the ARE in the enhancer (ARE III) is almost 4 kb away from ARE I and ARE II in the promoter, we asked how are the enhancer and promoter able to coordinate their activity? In order to answer this, we performed ChIP experiments to determine the dynamics of recruitment of AR coactivators, the association by RNA polymerase II, and the appearance of histone acetylation in the promoter region (primers A/B, Figure 6A) and the enhancer region (Primers G/H, Figure 6C). As a control, we used primers to a region located between the promoter and enhancer (primers E/F, Figure 6B). The recruitment of both GRIP1 and RNA polymerase II and the appearance of histone acetylation were detected at the promoter and enhancer but not in the nonfunctional region between them. Moreover, the dynamics of cofactor recruitment and histone acetylation was identical at the promoter and enhancer, suggesting that these elements act in concert to regulate PSA expression. This timing was consistent with the timing of the appearance of PSA mRNA (Figure 6D). The detection of RNA polymerase II associated with the enhancer strongly supports the notion that the enhancer is in close proximity to the transcription start site. Based on these results, we propose a model for the formation of an AR transcription complex (Figure 7) in which both the enhancer-bound AR and the promoter-bound AR interact with a common coactivator complex that contains p160 proteins, CBP, p300, and pCAF. According to this model, the local chromatin is modified and configured upon AR binding such that it brings the enhancer and promoter into close proximity, and the communication between the enhancer and promoter is mediated through the use of shared coactivator proteins.

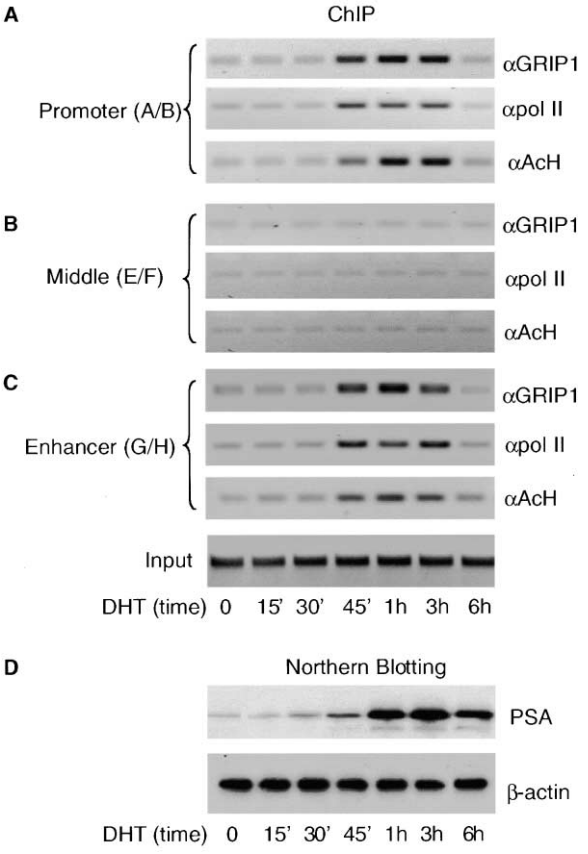

Figure 6. Coordination between the Enhancer and Promoter in the Assembly of the AR Transcription Complex

ChIP assays of the recruitment of GRIP1 and RNA polymerase II and histone acetylation to the promoter (A), enhancer (C), or nonfunctional region between the enhancer and promoter (B) of the PSA gene. LNCaP cells were treated with 10 nM DHT for the times indicated. Soluble chromatin was prepared from formaldehyde-cross-linked and sonicated cell cultures. Specific antibodies against GRIP1, pol II, and acetylated histones (AcH) were used to immunoprecipitate protein-bound DNA fragments. These fragments were amplified by PCR using the primer pairs described in Figure 2A. (D) Northern blot analysis of PSA gene expression. Total RNA was extracted, and Northern blotting was performed using exon 2 of the PSA gene as probe. β-Actin expression was used as the control.

Discussion

The analysis of the mechanism of ligand-dependent regulation of the PSA gene offers the opportunity to address the role of HATs and HDACs in mediating the activity of AR agonists and antagonists. In addition, it has revealed insights into how enhancer elements participate in the process of gene activation when separated from the transcription start site by thousands of nucleotides.

Chromatin modification by histone acetylation has been directly correlated with gene activation (Aalfs and Kingston, 2000; Glass and Rosenfeld, 2000; Pazin and Kadonaga, 1997). This is also the case for AR-mediated gene transcription. Coactivators involved in nuclear receptor-mediated gene activation include the histone acetylases CBP, p300, and pCAF (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996). In addition, the p160 family of coactivators has been reported to possibly possess weak intrinsic HAT activity as well (Chen et al., 1997; Spencer et al., 1997). Biochemical and genetic studies have demonstrated that the recruit-

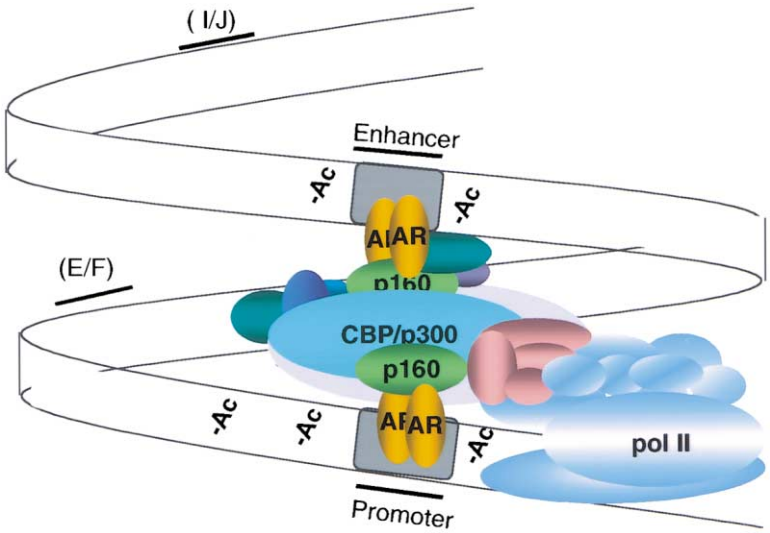

Figure 7. Model of the AR Transcription Complex

Agonist-bound ARs are recruited to both the enhancer and the promoter of the PSA gene. This is followed by a coordinated and ordered recruitment of p160 proteins, CBP, p300, and RNA polymerase II holoenzyme to form the AR transcription complex.

ment of these proteins during nuclear receptor-mediated gene transcription depends on the integrity of AF-2 (Durand et al., 1994; Halachmi et al., 1994; Jenster et al., 1997; Korzus et al., 1998; Lemon and Freedman, 1999; Wolffe et al., 1997; Xu et al., 1999). The existence of an independent AF-2 function for AR has been less well characterized, and thus, participation of a HAT complex in AR-mediated gene transcription has previously not been well established. Using ChIP, we found that p160, CBP, p300, and pCAF (data not shown) are indeed recruited by DHT-bound AR to the PSA gene. The dynamics of the recruitment of these proteins resembles that of ER-mediated gene transcription (Shang et al., 2000), except for a somewhat slower pace, with p160 and p300 recruited first, CBP second, and pCAF third. In addition, we found that the p160-CBP/p300 interface is crucial for AR-mediated gene activation, as deletion of AD1, the CBP/p300 interaction domain, abolished activity. This result is in agreement with previous studies suggesting that the p160 proteins mediate the major direct interaction between the HAT complex and the nuclear receptors and serve to link p300 and CBP to nuclear receptors (Chakravarti et al., 1996; Hanstein et al., 1996).

Antiandrogens, such as bicalutamide, have been successfully used as therapeutics for prostate cancers, as AR-mediated gene transcription plays an important growth stimulatory role. As is the case with antiestrogens, antiandrogens are believed to block gene activation by inducing a conformational change in AR that abrogates the binding of coactivator proteins (Matias et al., 2000; Poujol et al., 2000; Sack et al., 2001). In the absence of agonists, nuclear receptors such as RAR and TR are able to recruit a corepressor complex containing HDAC activities. Our previous study (Shang et al., 2000) and current experiments have demonstrated that both antagonist-bound ER and antagonist-bound AR are also able to recruit corepressors, suggesting that antagonism not only operates by blocking coactivator binding, but also includes an actively repressive activity. This raises the possibility that an as yet identified natural ER or AR antagonist may exist in vivo.

Three putative AREs have previously been identified in the androgen-responsive PSA gene; two are located in the proximal promoter region, whereas the third resides in the distal enhancer region. In response to DHT, AR is recruited to the AREs in both the enhancer and the promoter. In addition, coactivators are also associated with both the enhancer and the promoter in response to androgens. Interestingly, the formation of an AR corepressor complex appears to involve only the promoter, as AR and associated corepressors are found only at the promoter but not the enhancer in response to antagonists. This result suggests a distinct topology for the corepressor complexes.

Finally, we find that in addition to AR and associated coactivators, RNA pol II is recruited to both the promoter and enhancer, but not to sequences between them, in response to DHT. This result suggests that rather than being distant from the transcription start site, the enhancer and promoter are likely to be physically close together in the formation of a common AR-containing coactivator complex that we have termed the AR transcription complex (Figure 7). Such an edifice would be built by shared intermolecular interactions that facilitate and/or stabilize the formation of the AR transcription complex. This model provides a molecular basis for the need of AREs in both the promoter and the enhancer for maximal AR activity (Cleutjens et al., 1996, 1997a, 1997b; Huang et al., 1999; Reid et al., 2000).

A detailed mechanism underlying the different involvement of regulatory elements in AR-mediated gene activation versus repression remains to be determined. However, given that AR is detected at the enhancer only in response to agonists and not to antagonists, it is tempting to speculate that binding of AR to the enhancer is cooperative and requires components of the coactivator complex to mediate the effect.

Experimental Procedures

Materials and Reagents

Dihydrotestosterone (DHT) was purchased from Sigma (St. Louis, MO). Antibodies used in this experiment were αAR, PG-21 (Upstate Biotechnology, Inc., Lake Placid, NY); αPSA, MU014-UC (BioGenex,

San Ramon, CA); αAIB1 (affinity purified rabbit 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); αAcetylated histone (Upstate Biotechnology, Inc.); αNCoR and αSMRT (M. Lazar, University of Pennsylvania, Philadelphia, PA) (Huang et al., 2000); and αHDAC1 and αHDAC2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Plasmid Construction

The construction of a CoRNR box-containing GRIP1 (subGRIP1) was described elsewhere (Shang et al., 2000). subGRIP1 mutants were created by PCR (for AD2 deletion) or PCR with subsequent ligation of the N-terminal fragment and the C-terminal fragment (for AD1 deletion). The PCR primers were: AD1 deletion, N-terminal fragment: 5′ primer, GCGCGAATTCATGAGTGGGATGGGAGAAAAC, and 3′ primer, GCGCGGTACCGGCTGCCTGTTCTGGCT; C-terminal fragment: 5′ primer, GCGCGGTACCCTTCGGCAGCTCCCCTGAT, and 3′ primer, GCGCTCTAGAGCAGTATTTCCGAGATGCATCTC. subGRIP1 with AD2 deletion: 5′ primer, GCGCGAATTCATGAGTGG GATGGGAGAAAAC, and 3′ primer GCGCTCTAGACTGCTCCAGCA TTATGCTGGACT. The resultant products were inserted into Xba I and ECoR I sites of the pcDNA3.1(−) plasmid (Invitrogen, Carlsbad, CA).

ChIP

LNCaP cells were grown in RPMI-medium 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 5% charcoal-dextran-stripped fetal bovine serum. After 3 days of cultivation, cells were treated with appropriate ligands, washed with PBS, and cross-linked with 1% formaldehyde at 37°C for 10 min. Cells then were rinsed twice with ice-cold PBS, collected into PBS, and centrifuged for 5 min. The pellets were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and 1× protease inhibitor cocktail [Roche Molecular Biochemicals, Indianapolis, IN]) and sonicated three times at 15 s each at a submaximal input (Fisher Sonic Dismembrator, Model 300) followed by centrifugation for 10 min. Supernatants were collected and diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 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, and 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. Sepharose beads 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, and 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1). Beads 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 6 hr or overnight to reverse the formaldehyde cross-linking. DNA fragments were purified with a DNA purification kit (QIAquick Spin Kit, Qiagen, CA). For PCR, 1–5 μl out of 50 μl DNA extraction was used in 21–25 cycles of amplification. The primer sequences were as follows: A: TCTGCCTTTGTCCCCTAGAT, B: AACCTTCATTCCCCAGGACT; C: AGGGATCAGGGAGTCTCACA, D: GCT AGCACTTGCTGTTCTGC; E: CTGTGCTTGGAGTTTACCT GA, F: GCAGAGGTTGCAGTGAGCC; G CCTCCCAGGTTCAAGTGA TT, H: GCCTGTAATCCCAGCACTTT; I: GATGGTGTTTCACCGTGT TG, J: AGAGTGCAGTGAGCCGAGAT; and β-actin primers: forward: TCCTCCTCTTCCTCAATCTCG, reverse: AAGGCAACTTTCGGAACGG.

Northern Blotting

LNCaP cells were grown in phenol red-free RPMI medium 1640 supplemented with 5% charcoal-dextran-stripped fetal bovine serum for at least 3 days to 95% confluence and treated with DHT for different times. Total RNA was extracted with TRIZOL Reagents (GIBCO-BRL). The PSA probe was amplified exon 2 of PSA gene. A β-actin probe was from Oncogene Research (Cambridge, MA).

Reporter Assays

The reporter construct used was a 6 kb prostate-specific antigen gene regulatory region-driven luciferase (Cleutjens et al., 1997a). LNCaP cells were seeded in phenol red-free RPMI medium 1640 supplemented with 5% charcoal-dextran-stripped fetal bovine serum for 18 hr. Transfections were done using Fugene 6 Transfection Reagent (Roche Diagnostic Corporation, Indianapolis, IN). Forty-eight hours after transfection, cells were treated as indicated and luciferase activity was measured. Transfection efficiency was normalized with a β-galactosidase expression vector.

Western Blotting

Total cellular protein was separated on 7.5%–10% SDS-PAGE and transferred to nitrocellulose membranes which were then incubated with primary antibodies. After adding appropriate secondary antibodies, the blots were developed using an ECL kit (Amersham, Arlington Heights, IL).

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