Involvement of Proteasome in the Dynamic Assembly of the Androgen Receptor Transcription Complex*

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We have used the chromatin immunoprecipitation technique to analyze the formation of the androgen receptor (AR) transcription complex onto prostate-specific antigen (PSA) and kallikrein 2 promoters in LNCaP cells. Our results show that loading of holo-AR and recruitment of RNA polymerase II to the promoters occur transiently. The cyclic nature of AR transcription complex assembly is also illustrated by transient association of coactivators GRIP1 and CREB-binding protein and acetylated histone H3 with the PSA promoter. Treatment of cells with the pure antiandrogen bicalutamide also elicits occupancy of the promoter by AR. In contrast to the agonist-ligated AR, bicalutamide-bound receptor is not capable of recruiting polymerase II, GRIP1, or CREB-binding protein, indicating that the conformation of AR bound to anti-androgen is not competent to assemble transcription complexes. Proteasome is involved in the regulation of AR-dependent transcription, as a proteasome inhibitor, MG-132, prevents the release of the receptor from the PSA promoter, and it also blocks the androgen-induced PSA mRNA accumulation. Furthermore, occupancy of the PSA promoter by the 19 S proteasome subcomplex parallels that by AR. Collectively, formation of the AR transcription complex, encompassing AR, polymerase II, and coactivators, on a regulated promoter is a cyclic process involving proteasome function.

Androgens control a variety of developmental processes that create the male phenotype and are important for male fertility. Notably, androgens play a key role both in the development and maintenance of normal prostate and in the initiation and progression of prostate cancer, the most common male malignancy in the Western world (1, 2). The action of androgens is mediated through the androgen receptor (AR)† that belongs to the steroid hormone subfamily of nuclear receptors (3). These ligand-regulated transcription factors are composed of single polypeptides harboring three separable functional domains as follows: a relatively well conserved C-terminal ligand-binding domain, a highly conserved DNA-binding domain, and a poorly conserved N-terminal domain. In the absence of ligand, AR is complexed in the cytoplasm to chaperone proteins that keep the receptor in a transcriptionally inactive form. Upon binding the hormone, AR dissociates from the chaperones and translocates to the nucleus where it binds to androgen-response elements (ARes) (1, 2). Experiments performed under in vitro conditions have suggested that the ligand-induced conformational change enables the receptor to recruit coactivators and/or proteins of the general transcriptional machinery to target gene promoters (4–8). The best characterized coactivators include the steroid receptor coactivator (SRC) family members (SRC-1, SRC-2/glucocorticoid receptor-interacting protein 1 (GRIP1)/transcription intermediary factor 2 (TIF2), and SRC-3/activator of thyroid and retinoic receptor/amplified in breast cancer (AIB1), CREB-binding protein (CBP/p300), and CBP/p300-associated factor (PCAF). A growing list of recently discovered AR coregulators supports the notion that a complex network of proteins regulates transcription by androgens (9, 10).

Histone acetylation is a dynamic process directed by histone acetyltransferases and histone deacetylases, resulting in alterations in nucleosome structure (6, 11, 12). Acetylation of histone tails is thought to relax chromatin packaging and thereby facilitate gene transcription. Recruitment of complexes that affect acetylation of chromatin domains has been shown to be important for transcriptional regulation by steroid receptors (5–8). Many of the coactivator molecules, including PCAF, CBP/p300, and SRC-1, possess inherent histone acetyltransferase activity (5–8). Recent assays in vivo have revealed that holo-estrogen receptor (ER) α and several coactivators indeed assemble onto estrogen-responsive promoters in a cyclic fashion and in a specific order, indicating that promoter remodeling by histone acetylation is a dynamic and stepwise process (13). In addition to core histones, CBP/p300 and PCAF are capable of acetylating steroid receptors, such as AR and ER, and the coactivator SRC-3/activator of thyroid and retinoic receptor/AIB1 (14–16). In contrast to coactivators, corepressors bind to nuclear receptors in the absence of ligand, or in the presence of an antagonist, and recruit histone deacetylases, leading to condensation of nucleosomal structures and repression of transcription (6, 8).

AR is able to interact in vitro, in cell-free systems and in

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‡ The abbreviations used are: AR, androgen receptor; AcH3, acetylated histone H3; ARE, androgen-response element; CBP, CRE-binding protein-binding protein; CDX, casodex; ChIP, chromatin immunoprecipitation; ER, estrogen receptor; FCS, fetal calf serum; GRIP1, glucocorticoid receptor-interacting protein 1; KLK2, kallikrein 2; PCAF, p300/CBP-associated factor; pol II, RNA polymerase II; PSA, prostate-specific antigen; T, testosterone; CREB, cAMP-response element-binding protein; PMSF, phenylmethylsulfonyl fluoride; nt, nucleotide; snRNA, small nuclear RNA; SRC, steroid receptor coactivator.
transfection assays, with the general transcription factors TFIIH and TFIIIF and a large number of nuclear coregulatory proteins (9, 10, 17–20). However, the physiological significance of the majority of these interactions has remained elusive. Chromatin immunoprecipitation (ChiP) is a powerful technique to recognize endogenous transcription factors assembled onto gene promoters in vivo (21). To understand better AR-dependent transcription in vivo, we have performed ChiP assays in LNCaP cells using the prostate-specific antigen (PSA) as the main target promoter (22). Our results show that in androgen-treated LNCaP cells, loading of AR and recruitment of coactivators and pol II to the PSA promoter is a transient and cyclic event that involves hyperacetylation of core histones. Even though the anti-androgen bicalutamidetreated AR is able to associate with the promoter, it is incapable of recruiting pol II and coactivators. Both the cyclic nature of PSA promoter occupancy by AR and androgen-mediated induction of PSA mRNA accumulation are abolished by a proteasome inhibitor, indicating that proteasome function is critically involved in the regulation of AR-dependent transcription.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies—** AR antibody raised against full-length rat (r) AR has been described (23). Anti-pol II (N-20) antibody (sc-899) and anti-GRIPl monoclonal antibody were from Santa Cruz Biotechnology and Neomarkers, respectively. Anti-acetylated histone H3 antibody, anti-CBP antibody, and anti-proteasome S1 subunit antibody were purchased from Upstate Biotechnology, Inc. Testosterone (T) was from Makor Chemicals. Bicalutamide (casexodex, (2R,2S)-2’-hydroxy-2-methyl-3’-fluorophenyl-propionanilide) was a gift from Zeneca Pharmaceuticals. MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was from Sigma.

**Cell Culture—** LNCaP human prostatic carcinoma cells from American Type Culture Collection were maintained in RPMI 1640 medium with 10% fetal calf serum (FCS), 2 mm glutamine, penicillin (25 units/ml), and streptomycin (25 μg/ml) in a 5% CO₂ atmosphere at 37 °C. At ~50% confluency, medium was changed to RPMI 1640 containing 2% charcoal-stripped FCS for 4 days to reach 90% confluency. Medium was changed, and the cells were cultured for another 24 h prior to the exposure to T or bicalutamide (casexodex (CDX)) for various times before harvesting.

**Chromatin Immunoprecipitation Assay—** Chromatin was prepared from LNCaP cells (~1 × 10⁸ according to Nissen and Yamamoto (24). In brief, the cells were fixed by adding formaldehyde to the medium to a final concentration of 1%. After cross-linking for 10 min at 22 °C, glycine was added to a final concentration of 125 mM, and the cells were rinsed with PBS, harvested into lysis buffer (50 mM Heps-KOH, pH 8.0, 1 mm EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100, 1 mm PMSF, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin), and natted for 10 min at 4 °C. Lysates were centrifuged, resuspended in wash buffer (10 mM Tris-HCl, pH 8.0, 1 mm EDTA, 0.5 mM EGTA, 200 mM NaCl, 1 mm PMSF, and 5 mg/ml each of leupeptin, pepstatin A, and aprotinin), and natted for 10 min at 4 °C. Resulting nuclei were centrifuged and resuspended in RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mm EDTA, 0.5 mM EGTA, 140 mM NaCl, 1 mm PMSF, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin). Chromatin was sonicated to an average DNA length of 500–1000 bp using Fibra Cell 375W Sonicator with a microtip (6 × 10 s at maximum power). Sonicated samples were centrifuged, precleared by incubation with normal rabbit serum and protein G beads, and subjected to immunoprecipitation with specific antibodies in the presence of 100 μg/ml of sonicated salmon sperm DNA (Sigma) with rotation overnight at 4 °C. Immunocomplexes were collected by adsorption onto protein G beads, and the beads were washed sequentially with TSE I (0.1% SDS, 1% Triton X-100, 2 mm EDTA, 20 mm Tris-HCl, pH 8.1, and 100 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 mM LiCl, 1% Nonidet P-40, 0.5 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were washed three times with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mm EDTA), and antibody-bound chromatin fragments were eluted from the beads with 1% SDS in 0.1 N HAcO₃.

Cross-links were reverted by heating at 65 °C overnight. DNA was recovered using QIAquick PCR purification system (Qiagen) and analyzed for PSA, kallikrein 2 (KLK2), U6 snRNA, and HSP70 gene sequences by using PCR.

**PCR Analysis of Immunoprecipitated DNA—** PCRs were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.). Control reactions with genomic DNA were always carried out alongside the immunoprecipitated samples. For amplifying PSA and KLK2 gene fragments, 25–38 PCR cycles were used. Each cycle consisted of a 45-s denaturation at 95 °C, a 45-s annealing at 60 °C, and a 45-s elongation at 72 °C. The following primers were used: PSA (–170→+19), 5’-AGAA-CAGCAATGCTAGTC-3’ and 5’-AGGTGGTAACTGTTGGGCTG-3’; KLK2 (–218→97), 5’-CTCCACAGTCTACAGTATG-3’ and 5’-TTG-GCACCAGATGTCCAGC-3’. The PCR primers for U6 snRNA (–245/+85) and HSP70 (–153/+423) genes have been described (24). The PCR products were fractionated on agarose gels, stained with ethidium bromide, and quantified using the Kodak Image Station 440 CF system.

**Immunoblotting—** AR and S1 proteasomes subunit were immunoblotted from aliquots of LNCaP cell extracts (40 μg protein) resolved on SDS-PAGE. For detection of pol II, CBP, and GRIP, cell extracts were immunoprecipitated with specific antibodies (2 μg of antibody/2 mg of cell extract) prior to immunoblotting with the same antibody. Proteins were transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Biosciences), and the membranes were blocked and incubated with primary antibody overnight at 4 °C. The blots were washed and incubated for 2 h with secondary antibodies (1:5000). Immunoblots were detected with enhanced chemiluminescence Western blotting detection reagents from Amersham Bio- sciences and visualized using the Kodak Image Station 440 CF system.

**Northern Blot Analysis—** Total RNA from LNCaP cells was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA probe for human PSA mRNA (522 bp, corresponding to nt 114–635 of complete PSA cDNA, M27274) was labeled with digoxigenin (Roche Molecular Biochemicals). The ribosomal S9 protein mRNA probe (431-bp DNA fragment corresponding to nt 225–655 of human S9 mRNA, XM050589) was used to confirm equal loading and transfer of RNA samples.
PSA promoter is a specific and ligand-dependent event that occurs very rapidly, i.e. within 15 min after the addition of T in the LNCaP cell culture medium.

Kinetics of RNA Polymerase II and Coactivator Recruitment to the PSA Promoter—To study the assembly of the AR transcription complex onto the PSA promoter, recruitment of pol II together with GRIP1 and CBP coactivators was monitored at various times after T exposure. Specific antibodies were used to immunoprecipitate pol II, GRIP1, CBP, and acetylated histone H3 (AcH3), and the bound chromatin DNA fragments were amplified by PCR with PSA-specific primers. The results revealed that loading of holo-AR onto the PSA promoter was accompanied by a rapid recruitment of pol II and the coactivators to the transcription complex, and that histone H3 was concomitantly hyperacetylated (Fig. 2A). Promoter occupancy by AR and recruitment of pol II and coactivators were transient events with cycles of ~90 min, and the second wave of promoter occupancy was greater than the initial one (Fig. 2B). By contrast, histone acetyltransferases activity on the promoter seemed to reach the maximum already during the first cycle, as judged by the amount of acetylated histone H3 present on the promoter (Fig. 2A). When examined at 15-min intervals, GRIP1 and CBP bound to promoter concomitantly rather than sequentially. Immunoblot analyses showed that T treatment did not influence the amounts of GRIP1, CBP, and pol II proteins during the experiment, thus ruling out the possibility that the cyclic nature of AR transcription complex assembly was due to changes in protein levels.

KLK2 represents another androgen-responsive gene expressed in LNCaP cells (28–29). Loading of AR onto the KLK2 promoter occurred rapidly (within 15 min) and transiently, essentially in a fashion identical to that of the PSA promoter (Fig. 3). In fact, occupancy of both the KLK2 and the PSA promoter by AR was detectable already within 2 min after exposure of LNCaP cells to androgen. As was the case with the PSA promoter, recruitment of pol II to the KLK2 promoter displayed cycles of ~90 min in duration, indicating that the cyclic nature of AR transcription complex assembly is not specific for the PSA promoter.

Association of AR and pol II to the PSA Promoter in LNCaP Cells Treated with the Anti-androgen Bicalutamide—Bicalutamide (CDX) is a nonsteroidal anti-androgen that exhibits very little agonistic activity in LNCaP cells, despite the T877A mutation in the AR ligand-binding domain in these cells (30). That CDX was indeed an anti-androgen under our experimental conditions was confirmed by analyzing induction of PSA mRNA accumulation after a 24-h treatment with CDX alone or in combination with T. In agreement with previous reports (31, 32), CDX alone failed to increase PSA mRNA accumulation in LNCaP cells, and it blunted the action of 100 nM T already at 1 μM concentration. To investigate whether AR bound to CDX was capable of occupying the PSA promoter, LNCaP cells were treated with 100 nM T or 10 μM CDX, or their combination, for 30 or 60 min before the ChIP assay. Surprisingly, CDX-occupied AR was loaded onto the PSA promoter approximately as efficiently as the T-ligated receptor (Fig. 4). Moreover, despite the fact that CDX inhibited T action on PSA mRNA accumulation, there was no reduction in PSA promoter occupancy by

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Fig. 1. Analysis of the human PSA promoter occupancy by chromatin immunoprecipitation assay. A, schematic representation of the PSA promoter region analyzed by the ChIP assay. The localization of the PCR primers (arrow) and androgen-response elements (ARE) is shown. B, recruitment of AR to the PSA promoter. LNCaP cells were treated with 100 nM testosterone for 15 or 120 min before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with anti-AR antibody (αAR) or normal rabbit serum (NRS) and analyzed by PCR with PSA, U6 snRNA, or HSP70 gene-specific primers and agarose gel electrophoresis as described under “Experimental Procedures.” Input, DNA prior to immunoprecipitation.

Fig. 2. Transient loading of holo-AR and recruitment of RNA polymerase II and coactivators to the PSA promoter in response to testosterone treatment of LNCaP cells. A, LNCaP cells were incubated with 100 nM testosterone for indicated times before harvesting for ChIP assay. Chromatin samples were immunoprecipitated with anti-AR antibody (αAR), anti-pol II antibody (αPol II), anti-AcH3 (αAcH3), anti-GRIP antibody (αGRIP1), or anti-CBP (αCBP) prior to PCR with promoter-specific primers followed by agarose gel electrophoresis and ethidium bromide staining. Input, DNA prior to immunoprecipitation. B, relative amounts of PSA DNA immunoprecipitated with antibodies against AR and pol II after androgen treatment of LNCaP cells. DNA bands were quantified by using Kodak Image Station 440 CF, and the graphs represent relative AR and pol II occupancy (mean ± S.E.) from three independent experiments.

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2 A. Pirskanen, Z. Kang, O. A. Jänne, and J. J. Palvimo, unpublished observations.
AR when the cells were exposed concomitantly to T and CDX. In contrast to T treatment, pol II, GRIP1, or CBP was not recruited to the PSA promoter by the CDX-ligated AR. Thus, even though the CDX-occupied AR is capable of associating with the PSA promoter, the conformation of the anti-androgen-bound receptor does not permit the recruitment of the two coactivators and pol II to the promoter.

Effect of Proteasome Inhibition on the Loading of AR to the PSA Promoter—Many nuclear receptors are subject to degradation via the proteasome, and increased turnover of nuclear receptors and other transcription-regulating proteins is linked to transcriptional activation (33–39). Transcriptional activation domains of the proteins often serve as signals for ubiquitination, suggesting that the proteasome itself takes part in transcription (40–43). With regard to AR, we have observed that transcriptional activity of AR is coupled to agonist-induced ubiquitination of the receptor.3 Thus, degradation of holo-AR initially loaded onto a regulated promoter may be needed for the ensuing rounds of transcription. To examine whether the 26 S proteasome is involved in the activation of the PSA promoter, the proteasome inhibitor MG-132 was added to the culture medium 2 h before the exposure to T for various times. MG-132 did not inhibit occupancy of the PSA promoter by holo-AR (Fig. 5). By contrast, it prevented the release of the receptor from the promoter after the first cycle of loading (cf. Fig. 2 and Fig. 5A), implying that proteasome activity is needed for receptor release from the promoter. Treatment of LNCaP cells with MG-132 leads to an ~2-fold increase in the amount of immunoreactive AR, suggesting that the receptor is degraded via the 26 S proteasome (Fig. 5B). Proper function of the proteasome was not limited to the loading of AR onto the PSA promoter, because MG-132 treatment also abrogated androgen-induced accumulation of PSA mRNA (Fig. 5C) and KLK2 mRNA4 in LNCaP cells.

Proteins belonging to the 19 S regulatory subcomplex of the 26 S proteasome have been implicated in the regulation of transcriptional activators including nuclear receptors (8, 40). In addition, a 19 S proteasome subcomplex has recently been shown to be recruited to an activated promoter in yeast (44). Loading of proteasome complexes to the PSA promoter was examined by the ChIP assay with an antibody specific for the S1 subunit of the 19 S proteasome subcomplex (45). LNCaP cells were treated with MG-132 or T alone, or their combination, and chromatin samples were subjected to immunoprecipitation. T treatment resulted in a transient recruitment of the S1 subunit to the PSA promoter, and similar to its effect on AR release, MG-132 prevented the release of the S1 subunit after the first cycle of promoter loading (Fig. 6). Collectively, the proteasome appears to play an important role in AR-dependent transcription, and one of the processes regulated by the proteasome is the release of AR from the promoter.

DISCUSSION

In this work, we have shown that agonist-dependent loading of holo-AR and recruitment of coactivators and pol II to PSA and KLK2 promoters in LNCaP cells are transient and cyclic. Even though the anti-androgen bicalutamide-ligated AR was able to occupy the promoter, it was incapable of recruiting pol II and coactivators. Importantly, both the cyclic nature of PSA

3 S. Tian, O. A. Jänne, and J. J. Palvimo, manuscript in preparation.
mediator recruitment. Moreover, binding of p65/RelA to the translation is a prerequisite for thyroid hormone-associated protein.

...in vivo number of coactivators with the cathepsin promoter... Ordered recruitment of histone acetyltransferase complexes to the proximal promoter but not to the upstream enhancer region (ARE III).

Besides the factors of the general transcription machinery and coactivators with histone acetyltransferase or methyltransferase activity, several other proteins are known to interact with AR in vitro and activate AR function in reporter gene assays (9, 10). However, very little is known about the role of these factors, some of which are expressed in a cell-specific fashion, in the assembly or disassembly of AR transcription complexes. The potential cell type- and promoter-specific differences in the AR transcription complexes as well as characterization of the complexes during the postulated ligand-independent activation of transcription by AR (62) warrant further studies.

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Androgen-induced Transcription Complex

48371

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