p300 and p300/cAMP-response Element-binding Protein-associated Factor Acetylate the Androgen Receptor at Sites Governing Hormone-dependent Transactivation*

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The androgen receptor (AR) is a sequence-specific DNA-binding protein that plays a key role in prostate cancer cellular proliferation by dihydrotestosterone and the induction of secondary sexual characteristics. In this study we demonstrate that the AR can be modified by acetylation in vitro and in vivo. p300 and p300/cAMP-response element-binding protein acetylated the AR at a highly conserved lysine-rich motif carboxyterminal to the zinc finger DNA-binding domain. [14C]acetate-labeling experiments demonstrated that AR acetylation by p300 in cultured cells requires the same residues identified in vitro. Point mutation of the AR acetylation site (K632A/K633A) abrogated dihydrotestosterone-dependent transactivation of the AR in cultured cells. Mutation of the p300 CH3 region or the p300/cAMP-response element-binding protein histone acetylase domain reduced ligand-dependent AR function. The identification of the AR as a direct target of histone acetyltransferase co-activators has important implications for targeting inhibitors of AR function.

The androgen receptor (AR) is a member of a nuclear receptor superfamily that binds specific DNA sequences and regulates ligand-dependent gene transcription (1, 2). The functional domains of the AR (termed A-F) are conserved with other members of the “classical” receptor subclass, which includes receptors for estrogens, progestins, glucocorticoids, and mineralocorticoids. Nuclear receptors may direct the assembly and stabilization of a pre-initiation complex at a target gene promoter through forming direct associations with the basal transcription apparatus and with hormone-dependent cofactors (3). Although interactions between nuclear receptors and components of the basal transcription apparatus including the TATA-binding protein (TBP) and TAF130 (4) are necessary, additional coactivator proteins are required for efficient transactivation (5).

The coactivator proteins cAMP-response element-binding protein (CBP) and the related functional homologue p300 (6) regulate gene expression through several distinct mechanisms. p300/CBP may serve a bridging function between the DNA-bound transcription factor and the basal apparatus because p300/CBP interacts with TATA-binding protein (7), TFIIIB (8), and RNA polymerase II (9). Secondly, p300/CBP provides a scaffold interacting with numerous transcription factors through dedicated domains to assemble high molecular weight “enhanceosomes” (reviewed in Ref. 10). Thirdly, the coactivator proteins p300/CBP share the capacity to acetylate histones, which correlates with their transcriptional coactivator function (11). In these circumstances acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene (12–15). Separaable intrinsic and “associated” histone acetyltransferase (HAT) activity functions have been identified, and the p300/CBP-associated factor (P/CAF) can direct the associated acetylase function (16, 17).

More recently, p300/CBP was shown to acetylate non-histone proteins including transcription factors. Transcription factor acetylation can have divergent and context-dependent effects on DNA binding and function (18). p300/CBP regulated acetylation of the tumor suppressor p53 (19), the erythroid Kruppel-like factor (20), the erythroid cell differentiation factor GATA-1 (21), and HATs themselves (22) (reviewed in Ref. 23). The acetylation of these transcription factors enhanced their activity. In contrast CBP repressed the transcriptional activity of T cell factor (24), and acetylation of HMG1(Y) by CBP decreased its sequence-specific DNA binding (25). In this report we demonstrate that AR activity is induced by either trichostatin A (TSA) (a specific inhibitor of histone deacetylase activity) or the HAT-containing transcriptional coactivators p300 and P/CAF. p300 and P/CAF acetylated the AR on conserved lysines carboxyterminal to the zinc finger DNA-binding domain. Mutation of these conserved lysines abrogated DHT-induced AR activity. Ligand-dependent AR activation is dependent upon acetylation at critical conserved lysines.

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¶ The abbreviations used are: AR, androgen receptor; CBP, cAMP-response element-binding (CREB) protein; MMTV-LUC, murine mammary tumor virus-luciferase; HAT, histone acetyltransferase; P/CAF, p300/CBP associated factor; DHT, dihydrotestosterone; GST, glutathione S-transferase; IP, immunoprecipitation; TSA, trichostatin A.
**AR Acetylation Regulates Ligand-dependent Transactivation**

**MATERIALS AND METHODS**

**Reporter Genes and Expression Vectors**—The expression vectors pCMSVHA-p300, pCMSVHA-p300Δ1737–1809 (26), pCApF, and P/CAF A690–624 (27) were previously described. The AR fusion proteins GST-AR(4676–919), GST-AR(505–637), GST-AR(505–559), GST-AR(550–633), GST-AR(1–614) (28), the Flag-tagged P/CAF mutants (29), Flag-p300(1135–2414), and the reporter MMTV-LUC (from Dr. R. Evans) were previously described. The wild type human AR was subcloned from pARO (30) into pcDNA3. The AR K632A/K633A and K630A mutant was derived by polymerase chain reaction-directed amplification using sequence-specific primers and cloned into pcDNA3. The integrity of all constructs was confirmed by sequence analysis.

**Cell Culture, DNA Transfection, and Luciferase Assays**—Cell culture, DNA transfection, and luciferase assays were performed as described previously (31, 32). The prostate cancer cancer cell line DU145 and the 293T cell line were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. The medium was changed after 5 h, and luciferase activity was determined after 24 h. At least two different plasmid preparations of each construct were used. In cotransfection experiments, a dose response was determined in each experiment with 300 and 600 ng of expression vector and the promoter reporter plasmids (2.4 μg). Luciferase activity was normalized for transfection using β-galactosidase reporters as an internal control.

Luciferase assays were performed at room temperature using an Autolumat LB 953 (EG & G Berthold) (31). The fold effect was determined for the empty expression vector cassette, and statistical analyses were performed using the Mann Whitney U test.

**Western Blots**—The antibodies used in Western blot analysis were anti-M2 Flag antibody (Sigma), guanine nucleotide dissociation inhibitor antibody (a generous gift from Dr. Perry Bickel, Washington University, St Louis, MO) (34) used as an internal control for protein abundance, antibodies from Santa Cruz Biotechnology for the AR in the presence or absence of TSA at the dose indicated for 24 h, C, cells co-transfected with the MMTV-LUC reporter and the expression vector for the AR were treated with DHT in the presence of an increasing dose of TSA. The data are mean ± S.E. for at least nine separate transfections. ARwt, wild type AR.

**FIG. 1.** AR function is regulated by the p300 HAT and trichostatin A. A, the MMTV-LUC reporter was co-transfected with expression vectors for the wild type AR, p300, or equal amounts of the empty expression vector cassettes (pCMV). Cells were treated with either DHT (10⁻⁷ m) or vehicle for 24 h, and luciferase activity was assessed. B, cells were co-transfected with the MMTV-LUC reporter and the expression vector for the AR in the presence or absence of TSA at the dose indicated for 24 h. C, cells co-transfected with the MMTV-LUC reporter and the expression vector for the AR were treated with DHT in the presence of an increasing dose of TSA. The data are mean ± S.E. for at least nine separate transfections. ARwt, wild type AR.
FIG. 2. The AR is acetylated by p300. A, schematic representation of the candidate acetylation motif identified in the AR in several species. Homology is shown with the acetylation motif of the murine GATA-1 and human p53 proteins. B, schematic representation of the human AR indicating the DNA binding domain (DBD), the ligand binding domain (LBD), the conserved RAXKK motif (indicated by asterisks), and the GST-AR fusion proteins. C, immunoprecipitation histone acetyltransferase assays were performed as described previously (12, 35). The GST or GST-AR fusion proteins were incubated with p300 and [3H]acetyl-CoA as detailed under “Materials and Methods.” The autoradiograms of the acetylated AR fusion proteins are indicated by the asterisks. D, the GST-AR fusion proteins or equal amounts of GST were incubated with p300 at 30 °C for 1 h, the products corresponding to the expected molecular weight were excised, and HAT activity was quantitated by Cherenkov counting, displayed in E. In F AR Western blotting was performed. 293 cells transfected with the AR were either subjected to directed Western blotting (lanes 1 and 2) or first immunoprecipitated using an AR-specific antibody (lanes 3 and 4) or an anti-acetyl lysine antibody (lanes 5 and 6). Comparison is shown with an IP performed using an equal amount of control IgG (lane 7). Cell extracts were either with (+) or without (−) DHT (10−7 M). hAR, human AR; rAR, rat AR; mAR, mouse AR; mGATA-1, mouse GATA-1.

was aspirated, and the cells were resuspended in 300 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol). The lysis mixture was incubated on ice for 30 min to extract the whole cell proteins and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant protein concentration was determined by the Bio-Rad assay. For immunoprecipitation the protein concentration was adjusted to 1 μg/ml in 500 μl. The relevant antibodies from Santa Cruz Biotechnology (p300, N15) were added (2 μg/500 μg of extract) and incubated at 4 °C for 2 h. Protein A-agarose beads (1:1 mix, 30 μl) were added. The mixture was rotated overnight at 4 °C. The complexed antibodies and associated proteins were pelleted and washed three times with lysis buffer. The beads were washed with HAT buffer (35) (1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl (pH 8.0), 10% glycerol, 10 mM butyric acid, 0.2 mM EDTA, 1 mM dithiothreitol), and a standard HAT assay was performed containing 5 μg of substrate and enzyme (either 200 ng of purified histone acetyltransferase (purified baculovirus p300 or P/CAP) or immunoprecipitated p300 from cultured cells (12, 35)). The mixture was incubated at 30 °C for 1 h. 90 pmol of [3H]Acetyl-CoA reaction were subjected to SDS-polyacrylamide gel electrophoresis and viewed following autoradiography of the gel. In addition, [3H]Acetyl incorporation into the substrates was determined by liquid scintillation counting. The reaction mixture was spotted onto Whatman P-81 phosphocellulose filter paper. The filter paper was air-dried for 2–5 min and washed with 0.2 M sodium carbonate buffer (pH 9.2) at room temperature with five changes of the buffer for a total of 30 min. The dried filter paper was counted in a liquid scintillation counter.

In vitro protein-protein interactions were performed as described (36). The in vitro translated protein (15 μl of AR, 1 μg of rabbit anti-AR polyclonal antibody (N20), and 5 μg of purified Flag-tagged baculovirus-expressed P/CAP were incubated in 300 μl of binding buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin) and rotated for 2 h at 4 °C. The mixture was incubated with 30 μl of protein A-agarose beads and rotated overnight at 4 °C. In the polypeptide competition experiments, the AR1 peptide or control peptide (1 nm) was co-incubated with the AR-P/CAP mix. Beads were washed four times with 1 ml of binding buffer, and 30 μl of binding buffer were added after the final wash. 6 μl of 6× SDS loading buffer were added, and the sample was denatured at 95 °C and subjected to electrophoresis on an 8% SDS-polyacrylamide gel. The gel was fixed with 25% isopropyl alcohol and 10% acetic acid for 15 min, washed with Amplify (Amersham Pharmacia Biotech) for 30 min to enhance the βC signal, and dried for 1 h at 80 °C, and autoradiography was performed at −70 °C using XAR ALF 2025 autoradiography film (LabScientific Inc., Livingston, NJ). The radioactivity was quantitated using a Molecular Dynamics phosphorImager and the ImageQuant software.

Mapping the AR Acetylation Sites—In vitro acetylation assays were performed as described previously (37). A synthetic peptide corresponding to the androgen receptor (AR1 residues 623 to 640), NH2-GMTLKKLGNLKLQ-OH, and a control polypeptide, NH2-ELVHMINWAKRYPGFVDD-OH, were synthesized by Biosynthesis (Lewisville, TX). The peptide was acetylated in vitro by incubating with 5 mM acetyl-CoA and baculovirus-purified Flag-p300 at 30 °C for 1 h. After incubation acetylated peptides were separated from contaminating p300 by passage through a 10-micron filter (Amicon) and further purified by analytical reversed phase high pressure liquid chromatography. The reaction products were analyzed with a PE Biosystems DE-STR matrix-assisted laser desorption ionization time of flight mass spectrometer. Further analysis by Edman degradation was performed on a PE-Biosystems Procise sequencer. The amount of phenylthiohydantoin-acetyl-lysine was measured by absorbance at 259 nm.

RESULTS

Activity of the AR Is Regulated by TSA and p300—Previous studies demonstrated that CBP induced AR activity in CV1 cells (38). We examined the role of p300 in inducing AR activity in cultured prostate cancer cells. p300 augmented AR activity
AR-(623–640) was acetylated in vitro A zinc finger DNA-binding domain. Approximately 2-fold (Fig. 1) p300 augmented DHT-induced AR activity approximately 20-fold. As shown in Fig. 1, TSA enhanced activity of the MMTV-LUC reporter and AR were treated with the ligand for a maximum of 4-fold (Fig. 1B) and did not affect the activity of the AR-(623–640) was acetylated by baculovirus p300. The acetylated product was subjected to Western blotting using the AR-specific antibody (Fig. 2A). An AR-immunoreactive band was identified in the anti-acetyl-lysine antibody IP of identical extracts from cultured cells, and acetylation was not observed using equal amounts of GST AR-(503–637) and GST AR-(1–614) and GST AR-(503–637). The [14C]acetate incorporation for each fusion protein was measured by the filter assay (Fig. 2C). Acetylation was not observed using equal amounts of GST protein (Fig. 2C, lane 1). The GSTAR-(1–614) and GSTAR-(676–919), which did not include the putative acetylation site, were not acetylated by p300 (Fig. 2D). The [14C]acetate incorporation for each fusion protein was measured by the filter binding assay (Fig. 2E). To determine whether the AR was acetylated in vivo, immunoprecipitation was performed using an anti-acetyl lysine antibody on cell extracts derived from cells transfected with the human AR expression vector (41). The immunoprecipitate was subjected to Western blotting with an AR-specific antibody (Fig. 2F). An AR-immunoreactive band was identified in the anti-acetyl-lysine antibody IP of identical

2- to 3-fold. The addition of 10−7 M DHT induced AR activity 7.5-fold. p300 augmented DHT-induced AR activity approximately 2-fold (Fig. 1A)

Fig. 3. p300 acetylates human AR carboxyl-terminal to the zinc finger DNA-binding domain. A, the peptide corresponding to AR-(623–640) was acetylated in vitro using 14C-labeled acetyl-CoA and p300. The acetylated product was subjected to electrophoresis on a 10–20% Tris glycine gel (Bio-Rad). B, a parallel reaction using unlabeled acetyl-CoA was analyzed by matrix-assisted laser desorption ionization mass spectrometry. The resulting peptide mass spectrum is shown. The major peak (X) corresponds to the expected mass of the unmodified AR peptide. The major peak labeled Y, larger by 42 mass units, represents singly acetylated peptide. No diacetylated or higher acetylated forms were present. The minor peaks are methionine oxidation products present in the starting material.

Regulation of transcription of certain promoters can be due to p300/CBP acetyltransferase activity (39). To determine whether acetylation plays a role in regulating the activity of the androgen-responsive enhancer in prostate cancer cells, we assayed the sensitivity of the MMTV-LUC to trichostatin A, a specific inhibitor of deacetylase activity (40). Briefly, the MMTV-LUC was co-transfected with an expression vector for p300. The acetylated product was subjected to electrophoresis on a 10–20% Tris glycine gel (Bio-Rad). As shown in Fig. 1B, TGA enhanced activity of the MMTV-LUC reporter in a dose-dependent manner to a maximum of 4-fold (Fig. 1B) and did not affect the activity of the AR-(623–640) (data not shown). Significantly, a more dramatic effect of TGA was seen when cells transfected with the MMTV-LUC reporter and AR were treated with the ligand for AR, DHT (Fig. 1C). In this case, the activity of the MMTV-LUC reporter was superinduced by TGA by approximately 20-fold, thus implying that DHT is rate-limiting in vivo. Together, these results indicate that the activity of endogenous deacetylase(s) might constrain the achievement of full activation potential by AR-regulated promoters.

p300 Acetylates the AR at a Region Containing a Conserved Lysine Motif—These studies suggested that DHT-induced AR activity is regulated by both endogenous histone deacetylase activity and by acetyltransferase containing transcriptional coactivators. Because p300 can regulate transcription factor activity through direct acetylation (18–21), we scrutinized the amino acid sequence of the AR for potential acetylation sites. Residues 623–640 are homologous to a RXKK motif acetylated by p300 in p53 and GATA-1 (Fig. 2A). The AR RXKK motif is highly conserved between different species and is located just carboxyl-terminal to the zinc finger DNA-binding domain. To determine whether the AR could be acetylated by p300 in vitro, purified GST-AR fusion proteins were exposed to p300 immunoprecipitated from cultured cells, and in vitro acetylation assays were performed (12). Strikingly, endogenous p300 acetylated the AR fusion proteins, which included the acetylation site (GST-AR-(503–637) and GST-AR-(550–833)) (Fig. 2C). Acetylation was not observed using equal amounts of GST protein (Fig. 2C, lane 1). The GSTAR-(1–614) and GSTAR-(676–919), which did not include the putative acetylation site, were not acetylated by p300 (Fig. 2D). The [14C]acetate incorporation for each AR fusion protein was measured by the filter binding assay (Fig. 2E). To determine whether the AR was acetylated in vivo, immunoprecipitation was performed using an anti-acetyl lysine antibody on cell extracts derived from cells transfected with the human AR expression vector (41). The immunoprecipitate was subjected to Western blotting with an AR-specific antibody (Fig. 2F). An AR-immunoreactive band was identified in the anti-acetyl-lysine antibody IP of identical
mobility to the band identified in the AR IP, whereas no AR immunoreactivity was observed in the IgG control IP. Together these studies suggest that the AR is modified by acetylation at lysine residues in cultured cells.

To identify the residues acetylated in the AR carboxyl terminus, a peptide, AR-(623–640), was synthesized and used as substrate for acetylation by p300 (Fig. 3A). Mass analysis confirmed the presence of two major ions differing by 42 mass units (Fig. 3B). The lower molecular weight product X corresponds to the unmodified AR peptide, whereas the higher molecular weight component Y corresponds to the acetylated AR product.

Mutation of the AR-conserved Lysine Residues (632/633) Abrogates p300 Acetylation in Vivo—To determine the functional significance of lysines 632/633, these residues were mutated to alanines in the context of the wild type AR to create the mutant plasmid AR-K632A/K633A. The wild type and mutant AR were transfected into AR-deficient cultured cells treated with DHT (10^-7 M), and Western blotting was performed for AR expression. Similar amounts of wild type and mutant AR were expressed as confirmed by normalization of transfer using the guanine nucleotide dissociation inhibitor antibody (Fig. 4A). The wild type and mutant AR were immunoprecipitated from transfected cells and used as substrates for in vitro p300-dependent 14C-acetylation assays. Equal amounts of wild type and mutant AR were identified by Western blotting of the AR immunoprecipitate (data not shown). Baculovirus-expressed p300 acetylated the wild type but not the AR K632A/K633A mutant. These studies demonstrate that the lysines 632 and 633 are required for full acetylation of the AR by p300 (Fig. 4B).

In previous studies of p53, acetylation analysis using Edman degradation had identified the capacity of lysines outside the RXXK motif to undergo in vitro acetylation, although mutation of these residues demonstrated redundancy and abrogated acetylation by CBP (21). Following in vitro acetylation of the AR peptide by p300, Edman degradation assays were performed. The results indicate that lysines 630, 632, and 633 were preferentially acetylated (Fig. 4C). Only monoacylated lysine-containing peptides were detected in the samples by matrix-assisted laser desorption ionization time of flight mass spectrometry, indicating that the product analyzed by Edman degradation was a heterogeneous population of peptides each acetylated at a single site. As mutation of lysines 632/633 completely abolished acetylation, it is likely that alanine mutations might prevent recognition of the acetylation motif by HAT or render lysine 630 inaccessible.

The P/CAF HAT Domain Is Required for Full Induction of AR Activity—p300/CBP and P/CAF have been shown to acetylate differentially select substrates (42). We therefore assessed p300 mutants that were either competent or defective in P/CAF binding for their effect on AR activity. The CH3 region of p300 interacts with P/CAF. Deletion of the CH3 region (Δ1737–1809) reduced p300 augmentation of AR activity by 30 to 40% (Fig. 5A). P/CAF has intrinsic HAT activity (16) located within a domain adjacent to the Ada2 homology region (Fig. 5B) (43). AR activity was induced by P/CAF overexpression; however, the P/CAF HAT domain deletion mutant (P/CAF Δ609–624) reduced AR-induced activity by 45% (Fig. 5B). The P/CAF wild type and mutant protein P/CAF Δ609–624 (ΔB) were expressed equally in DU145 cells by Western blotting using the anti-Flag antibody (data not shown).

P/CAF Binds and Acetylates the AR—To determine whether P/CAF bound AR and to determine the domains of P/CAF required for binding, assays were performed using affinity-purified P/CAF and in vitro translated AR (Fig. 6A). Detection of P/CAF and AR was performed by Western blotting. The AR bound to P/CAF (>5% of input, shown as +++) in Fig. 6A). An internal deletion mutant of the amino terminus Δ5–122 and deletions affecting the carboxyl terminus of P/CAF and the Ada2 homology region significantly reduced binding to the AR. Thus, the amino terminus and the carboxyl terminus including the bromo domain of P/CAF are required for full binding to the androgen receptor.

In vitro acetylation assays were performed using GST-AR and the baculovirus-expressed P/CAF. In the presence of GST only the 14C-labeled band corresponding to autoacylated

**Fig. 5.** P/CAF activates AR activity. 
A, schematic representation of the p300 expression vectors. The bromo, HAT, and CH3-binding domains are shown. The MMTV-LUC reporter was transfected into DU145 cells and co-transfected with the human AR and p300 as indicated. The data are shown as the mean ± S.E. DHT treatment (10^-7 M) was for 24 h. B, schematic representation of the P/CAF expression vector showing the histone acetyltransferase (HAT) domain and the regions homologous to the Ada2 and bromo domains. The P/CAF wild type or mutant expression vectors were co-transfected with the MMTV-LUC reporter in the presence of AR. DHT treatment was for 24 h. The data are the mean ± S.E. of five separate transfections. pCI, control vector for P/CAF.
P/CAF was observed. The GST-AR-(550–633) was strongly acetylated by P/CAF (Fig. 6B). The addition of DHT (10−7 M) did not affect [14C]acetyl-CoA incorporation into the AR. Because P/CAF bound and was capable of acetylating the AR, we examined the possibility that the peptide encoding the AR acetylation motif (AR residues 623–640) could compete with P/CAF binding to the AR. The AR was synthesized in vitro and incubated with full-length baculovirus-produced P/CAF. Co-incubation was performed in the presence of either the AR peptide (623–640) or a polypeptide of equal size but incapable of being acetylated in vitro, as described under “Materials and Methods.” The AR-P/CAF co-incubated mix was subjected to IP with the AR antibody, the complex was subjected to SDS-polyacrylamide gel electrophoresis, and Western blotting was performed with an antibody directed to the Flag epitope of the P/CAF protein. P/CAF binding to the AR was reduced by pre-incubation with the AR polypeptide (Fig. 6C) but not by equal molar amounts of the control peptide (data not shown).

**Mutations of the AR Lysine 632/633 Abrogate p300 and P/CAF Activation of AR Function**—Together these studies demonstrate that the P/CAF HAT domain is required for induction of AR activity, that P/CAF binds the AR, and that P/CAF is sufficient for acetylation of the AR in vitro. To assess the role of lysines 632/633 in AR transcriptional regulation, the AR-responsive MMTV-LUC reporter was used in transient expression studies in the AR-deficient prostate cancer cell line DU145. As noted above (Fig. 4A), the expression of the AR K632A/K633A mutant was similar to wild type in these cells. Activation of the AR K632A/K633A mutant by DHT was substantially reduced compared with wild type (Fig. 7A, lane 5 versus lane 6). p300 overexpression induced basal AR activity. However, the effect of p300 on DHT-induced activity was reduced to 50% of wild type. This finding is consistent with a role for acetylation in ligand-induced activation of the AR. Co-transfection of P/CAF augmented DHT-induced AR activity from 5- to 7.3-fold (Fig. 7B, lane 5 versus lane 6). In contrast the AR K632A/K633A mutant was defective in P/CAF augmentation of DHT-induced activity (compare lane 4 with lane 8). Together these findings demonstrate that the acetylation-defective AR K632A/K633A mutant is reduced in DHT-induced function in cultured cells.

Edman degradation had identified residue 630 as a candidate for direct acetylation (Fig. 4C). Because these studies suggested that acetylation was linked to ligand-induced activation of the AR, a point mutation of residue 630 (AR K630A) was performed, and the effect of this substitution on ligand responsiveness was assessed. The AR K630A mutant was expressed equally with wild type by Western blotting of transfected cells (data not shown); however, the induction by DHT (10−7 M) was reduced by 95%. Together these studies suggest that each of the AR lysine residues acetylated in vitro contribute to full induction of AR activity by DHT.

**DISCUSSION**

In recent studies a small number of non-histone proteins including p53 (35, 44), GATA-1 (37), erythroid Kruppel-like
factor (20), Xenopus NF-Y (45), and the human immunodeficiency virus transactivator protein (46) were identified as HAT substrates. Acetylation enhanced the activity of these transcriptional regulators through a conserved acetylation motif. Transcriptional activity of the AR is regulated by coactivators with intrinsic HAT activity, and we identified the presence of a candidate acetylation motif within the AR (Fig. 1A). The formal possibility that the AR functioned as a direct substrate for histone acetylation to regulate ligand-dependent activity had not previously been examined. The current studies are the first to demonstrate a critical role for acetylation in ligand-induced activation of the AR.

The current study demonstrated that ligand-induced AR function was enhanced by p300 and by P/CAF in prostate cancer cell lines. The coactivators p300/CBP and the p300/CBP-associated factors such as P/CAF contain intrinsic HAT activity (12, 13). We found that p300 acetylated GST fusion proteins that included a conserved acetylation motif (RXKK) and failed to acetylate other regions of the AR expressed as GST fusions. Mass spectrometry confirmed that the AR polypeptide (residues 623–640) was acetylated in vitro by p300. Mutation of lysines 632 and 633 abrogated acetylation of the full-length AR protein. The RXKK motif of the AR is highly conserved between species (Fig. 2A). The site of acetylation in the AR resembles the carboxyl-terminal acetylation motif of p53 and the GATA-1 acetylation site. Point mutation of two lysines within the GATA-1 acetylation motif abolished in vitro acetylation. The AR acetylation motif is located immediately carboxyl-terminal to the zinc finger DNA-binding domain, and the GATA-1 acetylation motif is immediately amino-terminal to its zinc finger binding domain.

In the current study, p300 or P/CAF acetylated the AR in vitro. Co-transfection analyses, however, suggested that only the CH3 region of p300, which binds P/CAF, rather than the HAT domain was required for full augmentation of DHT-induced activity. P/CAF also enhanced DHT-induced AR activity, and the P/CAF HAT domain was required for full induction. These findings suggest that p300-associated P/CAF HAT activity may contribute to AR acetylation and augment DHT-induced AR function in cultured cells but that P/CAF-independent activities of p300 probably contribute to AR induction. Baculovirus-expressed P/CAF bound the AR in pull-down experiments and was sufficient for AR acetylation. Deletion analysis of P/CAF demonstrated a critical role for the amino terminus and the carboxyl-terminal bromo and Ada2 domains for binding to the AR. The AR peptide (AR-(623–640)), which was acetylated by P/CAF, also competed with P/CAF for binding to the AR, suggesting that the acetylated motif of the AR probably contacts P/CAF. The solution structure of the P/CAF bromo domain revealed an unusual left-handed up and down four helix bundle (47). Furthermore the bromo domain module was shown to specifically interact with acetylated lysines (47). Together with our study, these findings are consistent with a model in which the P/CAF bromo domain may contact the acetylated lysines of the AR.

The current study does not preclude the possibility that other HATs may also regulate AR acetylation and function. There is considerable substrate specificity among the different acetyltransferases. P/CAF does not acetylate GATA-1 (21), and the carboxyl-terminal acetylation site of p53 is preferentially acetylated by p300 compared with P/CAF (19). Acetylation, through neutralizing positively charged lysines, is predicted to affect conformation and thereby protein-protein or protein-DNA interactions. Acetylation of p53 by P/CAF or p300 enhanced DNA binding (35), whereas p300 acetylation of erythroid Kruppel-like factor did not alter its DNA binding properties (20). GATA-1 acetylation affected DNA binding in one study (37), but this effect was not observed in another study (21). P/CAF-mediated AR acetylation may enhance transcriptional activity by affecting DNA binding or interactions with other proteins.

In addition to post-translational modification by acetylation at lysines within residues 630–633 as demonstrated in this study, the AR is also phosphorylated (48). In the current study...
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TSAs augmented basal and DHT-induced AR activity, consistent with a model in which histone deacetylases repress AR function. Regulation of transcription by histone deacetylase inhibitors, such as TSA, is in part mediated through activation of serine/threonine protein kinases and phosphatases (49). Recent studies demonstrated that phosphorylation-acetylation cascades regulate p53 function in response to DNA damage (44). AR phosphorylation is ligand-regulated, and a phosphorylation-acetylation cascade regulating DHT-dependent AR function remains a formal possibility. Although speculative at this time, it is plausible that activity of other nuclear receptors may be governed through direct acetylation and phosphorylation.

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