Tip60 and Histone Deacetylase 1 Regulate Androgen Receptor Activity through Changes to the Acetylation Status of the Receptor*

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The androgen receptor (AR), a member of the nuclear hormone receptor superfamily, is thought to play an important role in the development of prostate cancer. The AR is a hormone-dependent transcription factor that activates expression of numerous androgen-responsive genes. Histone acetyltransferase-containing proteins have been shown to increase activity of several transcription factors, including nuclear hormone receptors, by eliciting histone acetylation, which facilitates promoter access to the transcriptional machinery. Conversely, histone deacetylases (HDACs) have been identified which reduce levels of histone acetylation and are associated with transcriptional repression by various transcription factors. We have previously shown that Tip60 (Tat-interactive protein, 60 kDa) is a *bona fide* co-activator protein for the AR. Here we show that Tip60 directly acetylates the AR, which we demonstrate is a requisite for Tip60-mediated transcription. To define a mechanism for repression of AR function, we demonstrate that AR activity is specifically down-regulated by the histone deacetylase activity of HDAC1. Furthermore, using both mammalian two-hybrid and immunoprecipitation experiments, we show that AR and HDAC1 interact, suggestive of a direct role for down-regulation of AR activity by HDAC1. In chromatin immunoprecipitation assays, we provide evidence that AR, Tip60, and HDAC1 form a trimeric complex upon the endogenous AR-responsive PSA promoter, suggesting that acetylation and deacetylation of the AR is an important mechanism for regulating transcriptional activity.

Prostate cell growth, development, and homeostasis are critically dependent upon the androgen receptor (AR), an androgen-responsive transcription factor that activates expression of target genes in response to hormonal signals derived from the testes. The AR is a member of the nuclear hormone receptor (NHR) family and, in common with other family members, is a modular protein composed of numerous independently functioning domains (1–3). Upon binding to androgens within the cytoplasm, the AR translocates to the nucleus (4) where it recognizes and binds specific promoter elements and activates transcription of target genes through the concerted action of two transcriptional activation domains, namely activation function-1 (AF-1) and -2 (AF-2) (5).

The AF-2 domain of NHRs plays a fundamental role in receptor-mediated transcriptional activation. Upon ligand-binding, the C-terminal AF-2 undergoes a shift in conformation generating a platform suitable for protein-protein interaction with co-activator molecules (6, 7). To date, numerous co-activator molecules have been identified that function to enhance the transcriptional potential of NHRs (8). The majority of co-activators identified share the capacity to elicit histone acetyltransferase (HAT) activity, a catalytic process heavily implicated in target gene activation via chromatin remodeling (9, 10). Of the identified HAT-containing co-activators, several have emerged to play significant roles in NHR activity, including the p160 (11–13) and CBP (CREB-binding protein)/p300 families (14, 15) and PCAF (p300/CBP-associating factor) (16).

Whereas histone acetylation is important for initiating and maintaining transcriptionally active genes, the recruitment of factors involved in deacetylating target promoters is deemed to be a requisite for gene silencing. Numerous co-repressor molecules have been identified, such as Sin3 (17) and SMRT (18), which play an active role in transcriptional repression by numerous transcription factors such as unliganded class II NHRs (19). These repressors are found in complex with histone deacetylases (HDACs); enzymes that actively reduce the level of histone acetylation. To date, three groups of histone deacetylases have been identified. The class I family is composed of 4 members, HDAC1–3 and HDAC8, and are homologues of the yeast protein RPD3 (20). Six class II HDACs have been characterized, HDAC4–7, -9, and -10, which bear significant homology to the HDAC protein of yeast (20). Class III HDACs are homologous to the yeast Sir2 protein, but as yet, are not well characterized.

The finding that several transcription factors, such as p53 (21, 22) and MyoD (23, 24), are targets for direct acetylation and deacetylation suggests that factor acetyltransferase (FAT) and HDAC proteins, respectively, play an active role in regulating transcription factor function, in which the status of acetylation at both the histone and transcription factor level heavily influences gene expression profiles.

Recently, the AR has been found to be a substrate for p300- and PCAF-mediated FAT activity (25). Acetylation of three lysine residues in the short lysine-rich motif KKKK, flanking the DNA-binding domain (DBD), increases transcriptional activity of the AR, implicating this post-translational modification as a mechanism for regulating AR activity.

Tip60 (Tat-interactive protein, 60 kDa) was first identified in complex with the Tat protein of human immunodeficiency virus-1 (26) and was later demonstrated to directly acetylate histones H2A, H3, and H4 via a C-terminal HAT domain (27).
We previously identified Tip60 as an AR-interacting protein and showed Tip60 to be a bona fide co-activator for the AR (28). Moreover, we have recently demonstrated that Tip60 is a class I NHR-specific co-activator implicating an important role for Tip60 in steroid hormone receptor function (29). To further define the role of Tip60 in AR-mediated gene expression, we provide evidence that Tip60 directly acetylates the AR in vivo, which is a requisite for Tip60-mediated AR co-activation. We next investigated the potential for HDACs to influence AR transcriptional activity. Here we demonstrate that the AR is specifically down-regulated by the histone deacetylase activity of HDAC1, the effect of which can be reversed by the HAT activity of Tip60. In mammalian two-hybrid and immunoprecipitation experiments, we show that HDAC1 interacts directly with the AR. Using chromatin immunoprecipitation assays, we demonstrate that Tip60 and HDAC1 associate with the endogenous AR-responsive PSA promoter in LNCaP cells, implicating an important physiological role for acetylation and deacetylation in AR regulation. Together, the data suggests that the acetylation status of the AR is a dominant factor in regulating transcriptional activity, and is the first evidence that HDAC1 can down-regulate a member of the class I nuclear hormone receptor family.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—The following plasmids have been described previously: pPSALuc, UASLTRLuc, pCMV-β-gal, pcDNAs-AR (28), p-mAR-DS (29), pBJS-FLAG-HDAC1 and pBJS-FLAG-HDAC1 ΔH164A (gifts from Richard Pestell, the Albert Einstein Cancer Centre, Albert Einstein College of Medicine) (25).

The full-length Tip60 construct was generated by PCR, incorporating TipF (ATGGCTACCAAGACAGACGTGAACAGCGGTTGGGGGGAGATAATCGG) (anneals to the start codon of Tip60 and incorporates a FLAG epitope) and TipR (TCAGCCATCTGTGCCAGTCATG) (anneals to the stop codon of Tip60), using POZ-Tip60 (gift from Yoshihiro Nakatani, Dana-Farber Cancer Research Institute) (25) as template and Bio-Taq DNA polymerase enzyme (Bioline). The product was cloned into the TA-vector (Invitrogen) and then subcloned into pCMV vector via the EcoRI site.

The Tip60 HAT-defective mutant, Tip60ΔH164A (gift from Tsuyoshi Ikura) (35) as template and Bio-Taq DNA polymerase enzyme, incorporating TipF and TipR. The product was cloned into the TA-vector and then subcloned into the pCMV vector via the EcoRI site.

To generate pVP16AD-HDAC1, PCR was performed with HDAC1F, GAATTCCATGGCGAGCGGTCGACCATGCCATGATCCTCTGACGGAAG (anneals to the start codon of HDAC1), and HDACR, GGATCCTCAGGCCAACTTGACCTCGCCTCGCCCT, and insert PCR-amplified DNA (anneals to the stop codon of HDAC1), incorporating pBJS-FLAG-HDAC1 as template and Bio-Taq DNA polymerase enzyme, incorporating TipF and TipR. The product was cloned into the TA-vector as system before and then subcloned into pVP16AD (CLONTECH), via the BamHI and EcoRI sites. All constructs were fully sequenced to confirm integrity. Tip60-specific antibody was generated by injecting rabbits with a Tip60 peptide (amino acids 283–297) and then the antibody was affinity purified.

**Cell Culture and DNA Transfection**—Cell culture and DNA transfection were performed as described previously (28). COS-7 cells were maintained in RPMI 1640 media containing 10% fetal calf serum (FCS) (Invitrogen), 1% penicillin, and 1% streptomycin. 1 x 10⁶ COS-7 cells were routinely plated per well in 24-well microtiter plates (Corning). After 24 h, the cells were transfected using Superfect (Qiagen) according to the manufacturer’s recommendations. After 24 h, cells were washed and incubated either in FCS-containing media prior to treatment with 100 nM trichostatin A (TSA), or in RPMI 1640 media containing 10% FCS that had been stripped of steroids by treatment with dextran-coated charcoal prior to experimentation with 10 nM R1881 (synthetic androgen analogue). After 48 h, cells were harvested and assayed for luciferase activity according to the manufacturer’s guidelines (Promega).

Luciferase activity was corrected for the corresponding β-galactosidase activity to give relative activity as described previously (28). In general, TSA treatment lasted 12 h prior to cell harvesting, whereas treatment with R1881 lasted the duration of transfection.

The prostate cancer cell line LNCaP was cultured as above. For transfections, a superfect/DNA ratio of 4:1 for COS-7 cells was increased to 5:1 for LNCaP cells and the incubation period for transfection mixtures was increased from 2 h for COS-7 cells to 3 h for LNCaP cells.

In general, co-transfection experiments using both COS-7 and LNCaP cells incorporated 50 ng of each expression vector and 200 ng of each reporter construct. Fold increases were determined for 50 ng of each vector by comparing the activity with empty pcDNA3 Vector. Each experiment was performed in triplicate and repeated a minimum of three times.

**Western Blotting**—COS-7 cell lysates were boiled in SDS sample buffer (100 mM dithiothreitol, 125 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.005% bromophenol blue) for 10 min and equivalent amounts of protein were resolved on 12% polyacrylamide gels. Proteins were subsequently transferred to Hybond N+ membrane (Amersham Biosciences) and detected by specific antibodies (see Figs. 1, 3, and 6) using the ECL system (Amersham Biosciences) according to the manufacturer’s recommendations.

**In Vivo βH Labeling and AR Immunoprecipitation**—COS-7 cells were transfected with 5 μg of pcDNAs-AR and 5 μg of pCMV-Tip60 or empty pCMV vector. 1 h prior to harvesting, cells were incubated in FCS-containing media supplemented with 100 nM TSA and 1 μM [3H]acetic acid (ICN). Samples were subjected to immunoprecipitation as described in Ref. 28, using a polyclonal anti-AR antibody (Santa Cruz). Immunoprecipitates were resolved on a 12% polyacrylamide gel, soaked in Amplify (Amersham Biosciences), and then exposed to x-ray film at ~80 °C for 72 h.

To determine a direct role for Tip60 HAT activity in AR acetylation, COS-7 cells were transfected with 2 μg of pcDNAs-AR, pCMV-Tip60, or pCMV-Tip60ΔH164A per 90-mm dish and lysates were subjected to immunoprecipitation using a polyclonal anti-AR antibody (as described before) and immunoblotting with an anti-acetyllysine antibody (Upstate Biotechnology) to detect the acetylated AR species. To examine a potential AR-HDAC1 interaction, COS-7 cells transfected with 2 μg of pcDNAs-AR and pBJS-FLAG-HDAC1 per 90-mm dish were subjected to immunoprecipitation as described before using an anti-FLAG antibody to immunoprecipitate HDAC1-associated complexes and immunoblotting using a polyclonal anti-AR antibody.

**Chromatin Immunoprecipitation Assays**—LNCaP cells were grown on 150-mm dishes in FCS-containing media for 2 days until ~5 x 10⁶ cells were present. 16 h prior to androgen treatment, cells were transferred to steroid-depleted media (RPMI supplemented with 10% dextran-coated charcoal-stripped FCS). After 16 h, the media was replaced with FCS-striped media supplemented with or without 10 nM R1881 for the specified time period (see Fig. 8). Following treatment, LNCaP cells were treated with formaldehyde, added directly to culture medium to a final concentration of 1%, at room temperature for 10 min to cross-link histone proteins to DNA. Soluble chromatin was made as follows: cells were washed and detached by scraping following addition of ice-cold phosphate-buffered saline supplemented with 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 25 μg/ml pepstatin, and pelleted by centrifugation for 4 min at 700 x g. The latter two steps were repeated. The cell pellet was then subjected to immunoprecipitation by resuspending in lysis buffer (50 mM Tris (pH 8.1), 1% SDS, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.8 μg/ml pepstatin, 0.6 μg/ml leupeptin, and 0.6 μg/ml aprotinin), followed by sonication. Samples were then centrifuged at 13,000 rpm for 10 min and the supernatant was decanted and diluted 10-fold in dilution buffer (25 mM Tris (pH 8.1), 140 mM NaCl, 2% SDS, 3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.8 μg/ml pepstatin, 0.6 μg/ml leupeptin, and 0.6 μg/ml aprotinin). To pre-clear chromatin solution, 60 μl of salmon sperm DNA/protein A-agarose beads (Upstate Biotechnology) was added to each sample and agitated for 30 min at 4 °C. Beads were pelleted by brief centrifugation and the supernatant was collected. For immunoprecipitation, 2 μg of polyclonal AR antibody, monoclonal HDAC1 antibody (Upstate Biotechnology), or Tip60 polyclonal antibody (see above) were added to 1 ml of the purified chromatin sample and incubated overnight at 4 °C. Immunoblots were then re-probed by adding 60 μl of salmon sperm DNA/protein A-agarose beads for 1 h at 4 °C with agitation. Beads were washed sequentially for 5 min each in 10 ml of TSE buffers I–III and TE (pH 8), as described previously (36). Immunocomplexes were eluted by adding 250 μl of elution buffer (1% SDS and 0.1% NaHCO₃) to beads and subsequently heated for 4 h at 64 °C to reverse formaldehyde-induced cross-links. DNA were then recovered by phenol/chloroform extraction, eth-
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**RESULTS**

**Tip60 Mediates Direct Acetylation of the AR in Vivo**—The AR has been demonstrated to be directly acetylated by p300 and PCAF. Acetylation of the AR was shown to enhance inherent transcriptional activity of the AR, suggesting that acetylation plays a significant role in AR regulation (25).

We previously identified Tip60 as a bona fide co-activator for the AR (28). Considering that Tip60 contains a HAT domain, which has been shown to acetylate free histones H4, H3, and H2A (27), we sought to examine if, like p300 and PCAF, Tip60 directly acetylated the AR to increase transcriptional activity. To determine whether the AR is a target for Tip60-mediated acetylation in vivo, COS-7 cells were transiently transfected with wild-type AR and either Tip60 or empty vector for control, and incubated for 1 h in [3H]acetic acid prior to immunoprecipitation with a monoclonal anti-AR antibody. The level of AR acetylation was determined by measuring [3H]acetate incorporation into the AR protein using autoradiography. Previous work has shown that addition of the HDAC inhibitor TSA greatly enhances AR activity, suggesting that the AR is a potential target for direct deacetylation and down-regulation by HDACs (25). We incorporated 100 nM TSA into our system to block the action of deacetylase enzymes. As shown in Fig. 1A, a, in the presence of Tip60, the level of AR acetylation was increased substantially over that in the absence of Tip60 (compare lanes 1 and 2), indicating that Tip60 may directly acetylate the AR, presumably through the activity of the HAT domain. Our results also indicate that in the absence of overexpressed Tip60, endogenous factors within COS-7 cells have the capacity to induce AR acetylation (lane 1). Whether this modification is through the HAT activity of endogenous Tip60, or other potential HAT-containing proteins, such as p300 and PCAF, remains to be determined. Using Western blotting, incorporating an anti-AR antibody, we confirmed that the difference in acetylation observed was not from variation in transfection efficiencies between the samples (Fig. 1A, b).

To establish that the HAT activity of Tip60 was responsible for directly acetylating the AR, the ability of wild-type Tip60, and a HAT-defective Tip60 mutant (Tip60Q377E/G380E) (25), to acetylate the AR was investigated. COS-7 cells transiently transfected with full-length AR and wild-type Tip60 or Tip60Q377E/G380E were immunoprecipitated using an anti-AR antibody followed by immunoblotting incorporating an anti-acetyllysine antibody, to compare the levels of AR acetylation in the presence of either wild-type Tip60 or Tip60Q377E/G380E. We found that if AR hyperacetylation is a result of Tip60-mediated HAT activity, then overexpression of the Tip60 HAT mutant, which lacks a functional HAT domain through the substitution of two residues (Glu-377 and Gly-380) required for acetyl-CoA binding (35), would fail to generate the acetylated form of the AR. As shown in Fig. 1B, in the absence of wild-type Tip60, no acetylated species of the AR was detected (lane 1), whereas in the presence of wild-type Tip60, the AR was clearly demonstrated to be in an acetylated form (lane 3). In contrast, overexpression of Tip60Q377E/G380E resulted in no change to the acetylation status of the AR (compare lanes 5 and 1), indicating that the HAT-defective Tip60 mutant is unable to acetylate the AR. Together, the data provide evidence that Tip60 is capable of directly acetylating the AR, and thus implicate Tip60 as a potential FAT protein.

**The HAT Activity of Tip60 and the KLKK Acetylation Motif of AR Are Necessary for Tip60-mediated AR Up-regulation**—We next sought to determine the functional significance of Tip60-mediated acetylation upon AR activity. The demonstration that the HAT domain of PCAF is important for up-regulating AR activity suggested that direct PCAF-mediated acetylation is a requisite for full induction of AR function (25). We therefore hypothesized that acetylation of the AR by the HAT domain of Tip60 is necessary to enhance transcriptional activity of the AR.

To test this, we compared the ability of wild-type Tip60 and a HAT-defective mutant Tip60 to stimulate AR-mediated gene expression. COS-7 cells were transiently transfected with AR and wild-type Tip60 or Tip60Q377E/G380E, together with the pPSALuc reporter that contains a 600-bp fragment of the androgen-responsive PSA promoter sequence upstream from the luciferase gene. As illustrated in Fig. 2A, in the presence of the synthetic androgen R1881, wild-type Tip60 augmented AR-mediated reporter expression 4.5-fold, whereas Tip60Q377E/G380E enhanced AR activity ~2-fold, implicating the importance of the HAT activity of Tip60 for full induction of the receptor. This result is in line with the previous report that AR induction was compromised in the presence of a HAT mutant of PCAF, which was suggested to be because of the failure for complete AR acetylation (25). Therefore, we speculate that Tip60-mediated AR acetylation contributes to the full induction of the receptor.

Edman degradation of a p300-acylated AR fragment, containing residues 623–640, revealed that the AR was preferentially acetylated on three lysine residues (Lys-630, Lys-632, and Lys-633) within the short KLKK motif of the hinge domain (25) (Fig. 2A). Substitution of these amino acids with alanine abolished AR acetylation and abrogated p300- and PCAF-me-
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mutants AR630 and AR632/633 are also shown. (HD), and KLKK, acetylation motif. The acetylation motifs of the AR mutants AR630 and AR632/633 are also shown. B, the effect of a HAT-defective Tip60 mutant, Tip60Q377/E380E, on AR activity was examined in COS-7 cells. Briefly, 50 ng each of pcDNA3-AR and pCMV-Tip60 or pCMV-Tip60Q377/E380E were co-transfected together with 200 ng of pPSALuc and pCMV-β-gal reporters as indicated, in the presence and absence of 10 nM R1881. After 48 h, cells were harvested and assayed for luciferase activity and corrected for β-galactosidase activity to give relative luciferase activity. C, to examine the effect of Tip60 on AR mutants AR630 and AR632/633, 50 ng of pCMV-Tip60, pcDNA3-AR, -AR630, and -AR632/633 were transfected into COS-7 cells together with 200 ng of both pPSALuc and pCMV-β-gal reporters per well, in the presence and absence of 10 nM R1881. Relative luciferase was determined as above.

To confirm the importance of Tip60-mediated AR acetylation for enhancement of AR transcriptional activity, we sought to determine the effect of Tip60 upon two mutant AR proteins which, through the substitution of lysine residues by alanine within the KLKK motif, do not undergo full acetylation. Using AR630, which lacks the Lys-630, and AR632/633, which lacks both Lys-632 and Lys-633 (Fig. 2A), we figured that Tip60, like p300 and PCAF, would fail to fully augment mutant AR-mediated gene expression and thus further implicate Tip60-mediated acetylation as important for AR induction. Briefly, wild-type AR and both AR mutants were transfected into COS-7 cells with wild-type Tip60 together with the PSA reporter, in the presence and absence of R1881. As shown in Fig. 2C, wild-type AR activity was activated 2-fold by R1881 (lane 2 versus lane 1), whereas both AR630 and AR632/633 were relatively unresponsive to androgen (lane 6 versus 5 and lane 10 versus 9), a result in line with a previous report (25). As expected, co-transfection of Tip60 augmented R1881-induced wild-type AR activity from 2- to 5-fold (lane 4 versus 2). In contrast, Tip60-mediated up-regulation of both AR630 and AR632/633 was -2-fold, indicating that the two AR lysine mutants do not undergo full co-activation by Tip60 (compare lanes 6 with 8 and lanes 10 with 12), a result similar to that previously found for p300 and PCAF (25).

Together, these findings implicate Tip60-mediated AR acetylation as an important step in up-regulating AR activity. Considering that Tip60 failed to enhance both AR mutants, which combined lack all three lysine residues, we speculate that the HAT activity of Tip60 may target each of the lysine residues of the KLKK sequence to generate the hyperacetylated, active AR.

HDAC1 Specifically Down-regulates AR Activity—The demonstration that HDACs down-regulate the transcriptional activity of numerous transcription factors, including MyoD (23) and p53 (22, 37), implicate deacetylation as a mechanism of transcriptional regulation. For example, the acetylated KSKK sequence of p53 is directly deacetylated by the class I deacetylases, HDAC1, -2, and -3 (22). Knowing that the AR is a direct target for acetylation, and shares a similar acetylation motif to p53, we hypothesized that AR is a potential target for HDAC activity. Indeed, the previous demonstration that AR activity is greatly up-regulated by the HDAC inhibitor TSA was the first evidence to suggest that AR activity may be effected, and down-regulated by HDACs (25). To assess the possibility of a role for HDACs in AR function, we determined the effect of various HDACs on transcriptional activity of the AR in reporter assays using the pPSALuc reporter. We figured that using a natural androgen receptor-responsive promoter element within the reporter, we would gain greater support to the notion that the effects observed in this experiment may be physiologically important. Briefly, members of both class I and II HDAC families were transiently transfected into COS-7 cells, maintained in FCS-containing media, in the absence and presence of AR, together with pPSALuc. As shown in Fig. 3A, in the absence of AR (open bars), class I and class II HDACs showed variable effects upon basal promoter activity, with only HDAC1 failing to influence basal reporter expression. It is interesting to note that HDAC3, -5, and to a lesser extent -6, up-regulated luciferase expression in the absence of the AR. Although these proteins may influence recruitment of another promoter-binding protein thus leading to a putative up-regulation of basal activity, it is likely to be an artifact of protein overexpression in our transfection system.

As expected, in the presence of AR (black bars), PSA pro-
moter activity was stimulated 5-fold over basal level in the absence of HDACs (compare lanes 1 and 7). In the presence of HDAC1 and -2, AR activity was repressed 5- and 6-fold, respectively (compare lanes 8 and 9 with 7), suggesting a potential down-regulatory role for these two deacetylases in AR-mediated transcription. Unlike HDAC1 however, the ability of HDAC2 to repress basal promoter expression implicates an indirect role for AR repression by HDAC2 and is likely to occur independently of the AR. In the presence of HDAC5 and -6, AR activity was not effected, whereas co-transfection of HDAC3 enhanced AR activity a further 1.6-fold. Overall, from the various deacetylases tested, the ability of HDAC1 to down-regulate AR activity without effecting basal promoter activity implicates HDAC1 as the sole repressor of AR activity upon the PSA promoter.

To eliminate the possibility that relative levels of AR are effected upon co-transfection of HDACs, therefore resulting in either reduced or enhanced transactivation, AR protein levels were determined following transient transfection experiments performed above, using an anti-AR antibody. As shown in Fig. 3B, AR protein levels in both the presence and absence of the various transfected HDACs were comparable, suggesting that variation in AR activity between the different samples is not through changes to receptor protein levels.

The Histone Deacetylase Activity of HDAC1 Is Required for Inhibition of AR Function—Down-regulation of p53 and MyoD is dependent upon the deacetylase activity of class I HDACs (22, 23, 37). The demonstration that AR-mediated transcription is repressed by HDAC1 also suggests an involvement of the deacetylase function of HDAC1. To test this, we incorporated deacetylase-defective mutant HDAC1H141A into transient transfection experiments and compared its effects upon AR against wild-type HDAC1.

As shown in Fig. 4A, reporter activity was up-regulated

Fig. 3. HDAC1 represses AR-mediated transactivation. A, the effect of class I and II HDACs on AR activity was assessed in transient transfection experiments in COS-7 cells maintained in FCS-containing media. DNA included 50 ng of each HDAC construct, 50 ng of pcDNA3-AR, and 200 ng each of the reporters pPSALuc and pCMV-β-gal per well. Relative luciferase activity was determined. B, cell extracts from A were immunoblotted with a monoclonal anti-AR antibody to determine relative protein levels of AR in each sample.

Fig. 4. The deacetylase activity of HDAC1 is required for AR repression. A, to determine the influence of HDAC1-mediated deacetylase activity on down-regulation of AR activity, the effect of the deacetylase-defective HDAC1 mutant, HDAC1H141A, on the AR was compared with that of wild-type HDAC1. COS-7 cells, maintained in FCS-containing media, were transiently transfected with 50 ng of pcDNA3-AR, 50 ng of both pJB5-HDAC1 and pJB5-HDAC1H141A, together with 200 ng of the reporters pPSALuc and pCMV-β-gal per well. Relative luciferase activity was determined as before.

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The Histone Deacetylase Activity of HDAC1 Is Required for Inhibition of AR Function—Down-regulation of p53 and MyoD is dependent upon the deacetylase activity of class I HDACs (22, 23, 37). The demonstration that AR-mediated transcription is repressed by HDAC1 also suggests an involvement of the deacetylase function of HDAC1. To test this, we incorporated deacetylase-defective mutant HDAC1H141A into transient transfection experiments and compared its effects upon AR against wild-type HDAC1.
in the presence of AR (lane 2 versus lane 1), whereas addition of wild-type HDAC1 resulted in an approximate 5-fold reduction in AR activity (lane 4 versus lane 2), without affecting basal promoter expression (lane 1 versus lane 3). HDAC1H141A stimulated basal reporter activity 3.5-fold (compare lanes 1 and 5); an effect similar to that observed with HDAC3 and HDAC5 (as shown in Fig. 3A). In the presence of AR, HDAC1H141A failed to down-regulate AR-mediated transactivation, but instead enhanced AR activity a further 2-fold (compare lanes 2 and 6). The inability of the deacetylase-deficient mutant to repress AR-mediated reporter expression therefore suggests that the deacetylase activity of HDAC1 is required to downregulate AR activity.

The phenomenon of HDAC1H141A-mediated reporter enhancement has also been reported in a similar study on the effects of deacetylation upon MyoD (23). In this case, HDAC1H141A increased both basal activity of the muscle creatine kinase promoter and the inherent activity of MyoD, suggesting that our result is not a promoter-specific nor AR-specific artifact.

To confirm the above observation, we incorporated the histone deacetylase inhibitor TSA into our transfection system containing both wild-type AR and HDAC1 together with the pPSALuc reporter construct. TSA has been demonstrated to reversibly repress enzymatic activity of members of the two HDAC families and has been used extensively to define the mechanism of action of numerous co-repressor molecules. We figured that if the deacetylase activity of HDAC1 is a requisite for AR repression, the addition of TSA should reverse HDAC1-mediated down-regulation of AR function and induce AR activity.

Previous reports have shown that TSA causes AR hyperactivity, which is suggested to be the result of the AR being in a hyperacetylated state (25). As depicted in Fig. 4B, the addition of 100 nM TSA caused an approximate 13-fold increase in AR activity confirming previous data. As before, in the presence of HDAC1, AR activity was down-regulated ~5-fold (lane 2). Upon addition of TSA, the repressive effect of HDAC1 was abolished, resulting in a 13-fold increase in AR activity (lane 4), comparable with the effect of TSA upon AR in the absence of HDAC1. Together, these experiments indicate that inherent deacetylase activity of HDAC1 is required for repression of AR-mediated transactivation.

HDAC1-mediated Down-regulation of AR Activity Is Dependent upon the Ligand-binding Domain of AR—We next sought to determine the domain of AR required for HDAC1-mediated repression. Considering that the acetylation motif of the AR is located within the hinge region, between residues 630 and 633, and direct acetylation of the KLKK motif by p300 and PCAF and potentially Tip60 (25) is deemed important for AR-mediated transcriptional activation, we hypothesized that HDAC1 could function through this region to induce AR down-regulation. To test this, we tethered a Gal4-DBD fusion containing both DBD and the ligand-binding domain of the AR (Gal4-AR-DBD) to the KLKK acetylation site, to a Gal4-responsive reporter (UASTKLuc) and tested the effects of HDAC1 upon inherent transcriptional activity of the truncated receptor in FCS-containing media.

As shown in Fig. 5, in the absence of AR-DS (−AR), both HDAC1 and HDAC1H141A showed minimal effects upon basal UASTKLuc reporter activity (lane 1 versus 2 and 3). Tethering Gal4-AR-DS to the UASTKLuc reporter (+AR-DS) resulted in a 2.5-fold induction of transcriptional activity (lane 4), indicating that the C-terminal portion of the AR, which contains the weakly active AF-2, retains the capacity to modulate gene expression. In the presence of HDAC1, but not the deacetylase

![Fig. 5](http://www.jbc.org/)
Fig. 6. AR and HDAC1 interact in vivo and in vitro. A, using the mammalian two-hybrid system, the potential of an AR-HDAC1 interaction was investigated. COS-7 cells were transfected with 50 ng of pm-AR-DS and 50 ng of the VP16AD fusion constructs pVP16AD-HDAC1, -Tip60, or empty pVP16AD for control, as well as 200 ng of UASTKLuc and pCMV-β-gal per well. Relative luciferase activity was determined. B, COS-7 cells were transiently transfected with 2 μg of pcDNA3-AR and pBBS-FLAG-HDAC1 per 90-mm dish. Cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody and immunoblotted with an anti-AR antibody. WB, Western blot.

The HAT Activity of Tip60 Abrogates the Repressive Effects of HDAC1 upon the AR—Because Tip60 and HDAC1 act to induce antagonistic effects upon AR-mediated transcription, we asked whether variation in the relative levels of these proteins influences the activity of the AR. Furthermore, to ascertain a role for acetylation/deacetylation in AR regulation, we incorporated increasing amounts of either wild-type Tip60 or Tip60Q377E/G380E, together with wild-type AR and pPSALuc reporter constructs, into our system. We hypothesized that whereas increasing amounts of wild-type Tip60 may overcome HDAC1-mediated repression through catalyzing AR hyper-acetylation, Tip60Q377E/G380E may be unable to reverse the effects of HDAC1 and thus implicate a potential role for deacetylation in AR silencing. COS-7 cells were transfected with HDAC1 and increasing amounts of either wild-type Tip60 or Tip60Q377E/G380E, together with wild-type AR and pPSALuc reporter constructs (Fig. 7). As expected, HDAC1 repressed AR activity ~3.5-fold (compare lanes 2 and 3). Co-transfection of 25 ng of wild-type Tip60 caused a negligible increase in AR-mediated transcription (lane 4), whereas transfection of 50 and 100 ng of Tip60 completely de-repressed the effects of HDAC1, reactivating the AR 3-fold over basal levels (compare lanes 5 and 6 with 1), indicating that increasing amounts of Tip60 can compete and overcome the effects of HDAC1. Unlike wild-type Tip60, however, increasing amounts of the HAT-defective Tip60 mutant failed to counteract the inhibitory effect of HDAC1 (compare lanes 7–9 with 3), implicating a role for acetylation in overcoming HDAC1 activity. Moreover, considering that the HAT activity of Tip60 is a requisite for abolishing HDAC1 activity, it is interesting to speculate that HDAC1 represses AR activity via direct deacetylation of the receptor.

HDAC1 and Tip60 Associate with the Endogenous PSA Promoter in LNCaP Cells—The finding that Tip60 and HDAC1 both bind AR and induce antagonistic effects upon the AR in reporter assays may suggest a role for these molecules in regulation of AR activity in the natural context. The recruitment of co-activators and co-repressors to target genes by various transcription factors has been shown to be a mechanism for controlling transcriptional activity. We therefore speculated that the AR may also have the potential to recruit both positive and negative regulating factors to responsive genes as a mechanism of transcriptional regulation.

To assess this, we used chromatin immunoprecipitation assays in AR-expressing LNCaP prostate cancer cells, incorporating antibodies specific for the AR, HDAC1, and Tip60, to examine the association of these proteins with the AR-responsive PSA promoter in the presence and absence of androgen (Fig. 8). A 20-min exposure of cells to androgen induced robust recruitment of endogenous AR to the PSA promoter (Fig. 8B, a, compare lanes 1 and 2), indicating that the AR, which we speculate is in an active state, rapidly associates with the target gene. Surprisingly, using anti-Tip60 and anti-HDAC1 antibodies, we detected a similar increase in promoter association of both Tip60 and HDAC1 20 min after hormone treatment (compare lanes 1 and 2 in Fig. 8B, b and c), suggesting that the two antagonists are recruited to the promoter within the same time interval. The correlation between AR and both Tip60 and HDAC1 for chromatin binding also implicates a potential role for these two proteins in regulation of AR activity. However, whether the proteins are active at the time of recruitment to the promoter will need to be addressed.

The previous finding that Tip60 up-regulates the activity of endogenous AR in LNCaP cells (28) suggests that the recruit-
sites, and bars nM R1881 were immunoprecipitated with antibodies against AR, soluble chromatin extracts from LNCaP cells treated with or without 10 nM R1881 were immunoprecipitated with antibodies against AR, HDAC1, and Tip60 followed by semiquantitative PCR incorporating primers specific for ARE2 in the PSA promoter. Input samples containing crude chromatin extracts prior to immunoprecipitation were also analyzed. C, to examine if HDAC1 down-regulated endogenous AR activity in LNCaP cells, transient transfection experiments were performed. DNA included 200 ng of pBBS-HDAC1 and 800 ng of the two reporters pPSALuc and pCMV-β-gal per well. Cells were maintained in FCS-containing media for 72 h prior to harvesting. Relative luciferase activity was determined.

Fig. 8. HDAC1 and Tip60 associate with AR on the PSA gene promoter under different hormone-induced states. To determine whether HDAC1 and Tip60 complexed with AR upon the PSA promoter in LNCaP cells, chromatin immunoprecipitation assays were performed. A, schematic representation of a fragment of the PSA promoter element containing androgen responsive element 2 (ARE2). Labeled arrows above and below the PSA promoter represent primer annealing sites, and bars and numbers indicate the length of PCR product. B, soluble chromatin extracts from LNCaP cells treated with or without 10 nM R1881 were immunoprecipitated with antibodies against AR, HDAC1, and Tip60 directly acetylates nucleosomal DNA on histone H4 (35), whereas p300 readily acetylates all four histones (15). We speculate that although each of the co-activators directly acetylate the AR to up-regulate activity in reporter assays, variation in histone substrate specificity between Tip60, p300, and PCAF may also aid in regulation of AR activity in the cell.

The finding that Tip60 directly acetylates the AR implicates Tip60 as one of a small subset of proteins, including PCAF and p300/CBP, capable of eliciting FAT activity upon a wide array of transcription factor targets. The small number of FAT proteins and the failure for other acetylases, such as members of the p160 family of co-activators, including SRC-1 and GRIP-1, to mediate acetylation of transcription factors is intriguing. Direct acetylation of target proteins is seemingly an initial event in transcription factor activation that precedes the subsequent binding of non-FAT acetylases, such as SRC-1 and GRIP-1, which act to increase transcription by eliciting histone acetylation. It may be that having a small number of potential co-initiator proteins, including p300/CBP, PCAF, and Tip60, serves to reduce the potential for other acetylase proteins to enhance transactivation of certain transcription factors and thus prevents spurious transcription factor-mediated gene expression.

Our studies identify the AR as the first FAT target for Tip60, however, the ability for Tip60 to enhance transcriptional activity of both estrogen and progesterone receptors to levels similar to AR (28), implicates a potential role for Tip60-mediated FAT activity upon these two nuclear hormone receptors, and may suggest an involvement of Tip60-mediated acetylation in diverse signaling mechanisms. Indeed, the recent demonstration that the HAT activity of Tip60 is required for stimulating UV irradiation-induced apoptosis in HeLa cells potentially broad-
ens the spectrum of proteins targeted for acetylation by Tip60 (35).

A better understanding of AR down-regulation came with the observation that HDAC1 specifically represses AR activity without effecting AR protein levels (Fig. 3). Our results indicate the importance of the deacetylase activity of HDAC1 for AR inhibition (Fig. 4) and suggest that HDAC1-mediated effects are potentially through direct deacetylation of the receptor (Fig. 7). Importantly, the demonstration that HDAC1 interacts with and inhibits the inherent activity of the DNA-binding and ligand-binding domains of the AR, which encompass the KLKK acetylation motif (Figs. 5 and 6), suggests that HDAC1 influences AR activity by binding to the AR. Whether the AR-HDAC1 interaction is direct or requires the assistance of one of a number of HDAC1-associated proteins, such as Sin3, remains to be determined. However, recent evidence shows that HDAC1 has the capacity to interact directly with both p53 and MxyO, without the presence of intermediary proteins (22, 23). Considering that the AR shares a similar acetylation sequence to p53, which is targeted by HDAC1, we hypothesize that HDAC1 recognizes and interacts directly with the AR, to reduce receptor activity, in a manner similar to that of p53.

Previous work has suggested that, unlike co-activator proteins, deacetylase enzymes exhibit minimal substrate specificity. The demonstration that p53 is repressed by HDAC1–3 upon the p53-responsive BAX promoter implicated redundancy in function between the three members of the class I HDAC family (22). However, our findings suggest otherwise, with both HDAC2 and -3, as well as the class II HDACs, failing to effect AR activity upon the AR-responsive PSA promoter. We hypothesize that like co-activator proteins, HDACs have a limited substrate specificity which is controlled by various determinants within target proteins, such as residues flanking the acetylation motif and/or the position of the target site within the substrate protein.

Mechanisms for AR down-regulation are as yet, poorly defined. Whereas the effect of receptor degradation on AR-mediated gene expression remains to be clarified, nuclear export of the active AR constitutes one mechanism for down-regulating the androgenic response. However, recent evidence has suggested that this process is only evident after 12 h in the absence of hormone (38), suggesting that it is unlikely to be the definitive mechanism for AR down-regulation in real-time. The ability of HDAC1 to repress AR activity indicates a novel mechanism for down-regulating AR-mediated transcription, which we speculate constitutes a more rapid mechanism for controlling the androgenic response. Indeed, the ability of HDAC1 to rapidly associate with the endogenous PSA promoter, which correlates with the recruitment of the AR to the promoter (Fig. 8B), suggests that HDAC1 has the potential to direct AR activity early in the transcriptional process. The failure for HDAC1 to reduce AR protein levels (Fig. 3B), combined with the ability of HDAC1 to down-regulate endogenous AR activity in LNCaP cells (Fig. 8C), implicates a role for HDAC1 in an acute and reversible mechanism for AR repression. We speculate that HDAC1-mediated deacetylation of the AR results in the generation of an inactive AR which has the capacity to be reactivated upon further ligand exposure and acetylation. Indeed, the demonstration that the AR undergoes both numerous cycles of activation and inactivation together with the respective nuclear-cytoplasmic shuttling, before succumbing to post-translational degradation (38), suggests that a mechanism of transient AR inactivation exists. Although these authors proposed that hormone inactivation or degradation may result in the transient deactivation of AR activity prior to receptor export from the nucleus, we suggest a role for HDAC1 in this system as changes to the acetylation status of the AR is a dominant factor in AR transcriptional activity.

The observations that HDAC1 and Tip60 have the potential to interact with the AR and are both associated with the AR-bound PSA promoter within the same time interval is suggestive of the existence of a trimeric complex containing the AR, Tip60, and HDAC1 upon the PSA promoter. The presence of both an acetylase and a deacetylase upon the same promoter was intriguing considering that they act to oppose the function of each other. Indeed, the demonstration that the HAT activity of Tip60 abrogated HDAC1-mediated repression of AR activity (Fig. 7) indicates competition between the two proteins; each sharing the ability to counteract the effect of the other upon the AR, potentially via acetylation and deacetylation. However, the recent demonstration that glucocorticoid receptor interacts with both p300/CREB and HDAC2 to repress p65-mediated granulocyte-macrophage colony-stimulating factor expression (39) suggests that the presence of antagonistically functioning proteins within the same complex is not uncommon and may arise to finely tune the transcriptional response. Also, the recent finding that both Tip60 and the class II HDAC, HDAC7, interact with the endothelin receptor, ETA (40), provides greater evidence that a co-activator and deacetylase protein can co-interact with a target protein. We speculate that the presence of HDAC1 on the promoter at the onset of the androgenic response may act to overcome or reduce Tip60-mediated effects upon the receptor and thus maintain the AR in a semi-active state, thereby preventing excessive androgen-responsive gene expression.

In summary, the results suggest the existence of a reversible and rapid mechanism for regulating AR activity that does not involve a reduction in AR protein levels: Tip60-mediated acetylation of the AR up-regulates the transcriptional response, whereas down-regulation of transcriptional activity occurs as a result of HDAC1, potentially via deacetylation of the AR. The ability of Tip60 to counteract the activity of HDAC1 suggests that the acetylation status of the AR is a dominant factor in AR functioning, and variation to the levels of FAT proteins and HDAC1 may give rise to fluctuating AR activity. Further investigation may focus upon the relative levels of these proteins in different stages of prostate cancer to identify if changes to the flux of acetylation and deacetylation of the AR exerts an influence upon cellular transformation.

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Acetylation and Deacetylation of the AR
Tip60 and Histone Deacetylase 1 Regulate Androgen Receptor Activity through Changes to the Acetylation Status of the Receptor
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