The Androgen Receptor Acetylation Site Regulates cAMP and AKT but Not ERK-induced Activity*

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The androgen receptor (AR) regulates ligand-dependent gene transcription upon binding specific DNA sequences. The AR conveys both trans-activation and trans-repression functions, which together contribute to prostate cellular growth, differentiation, and apoptosis. Like histone H3, the AR is post-translationally modified by both acetylation and phosphorylation. The histone acetyltransferase p300 transactivates the AR and directly acetylates the AR in vitro at a conserved motif. Point mutations of the AR acetylation motif that abrogate acetylation reduce trans-activation by p300 without affecting the trans-repression function of the AR. The current studies assessed the functional relationship between acetylation and phosphorylation of the AR. Herein trans-activation of the AR acetylation site mutants were enhanced by the p42/p44 MAPK pathway but were defective in regulation by protein kinase A (PKA) signaling. PKA inhibition augmented ARwt activity but not AR acetylation mutant gene reporter activity and association at an androgen response element in chromatin immunoprecipitation assays. Mutations of the lysine residues at the AR acetylation site reduced trichostatin A (TSA) responsiveness and ligand-induced phosphorylation of the AR. The AR acetylation site mutant formed ligand-induced phosphorylation-dependent isoforms with distinguishable characteristics from wild type AR as determined with two-dimensional electrophoresis. The AR conveys both trans-activation and trans-repression functions. Coactivators, including the p160 proteins, the p300/CBP, and the orthologue p300 (CBP/p300), form a complex. The C-terminal region of the AR, including the hinge region and ligand-binding domain (LBD) is responsible for ligand binding and dimerization. The well conserved DNA binding domain consists of 68 amino acids with two zinc finger structures. The N-terminal region contributes to transcriptional activation through its activation function 1 (2). In contrast to several other hormone-regulated nuclear receptors, the AR lacks an intrinsic activation function 2 function in the LBD. The LBD, which consists of twelve α-helices, projects away from the hormone-binding pocket in the absence of ligand and undergoes substantial conformational changes in the presence of ligand. The folding of the most C-terminal helix 12 (H12) over the ligand-binding pocket in turn creates new structural surfaces that bind coactivators required for efficient trans-activation.

Steroid receptors, including the androgen receptor (AR),1 are members of the nuclear receptor superfamily that generally function as ligand-dependent transcriptional regulators (1). In the absence of ligand, interactions with corepressors maintain the receptor in an inactive state. The AR is expressed in a variety of cell types and plays an important role in development, male sexual differentiation, and prostate cellular proliferation. The functional domains of the AR (termed A–F) are conserved with other members of the “classic” receptor subclass. The C-terminal region of the AR, including the hinge region and ligand-binding domain (LBD) is responsible for ligand binding and dimerization. The well conserved DNA binding domain consists of 68 amino acids with two zinc finger structures. The N-terminal region contributes to transcriptional activation through its activation function 1 (2). In contrast to several other hormone-regulated nuclear receptors, the AR lacks an intrinsic activation function 2 function in the LBD. The LBD, which consists of twelve α-helices, projects away from the hormone-binding pocket in the absence of ligand and undergoes substantial conformational changes in the presence of ligand (3). The folding of the most C-terminal helix 12 (H12) over the ligand-binding pocket in turn creates new structural surfaces that bind coactivators required for efficient trans-activation.

The AR conveys both trans-repression and trans-activation function. Coactivators, including the p160 proteins, the p300/CBP family, Ubc-9, ARA 70, ARA 55, BRCA1, and TIP60 (2, 4–8), appear to play cell type-specific effects on AR function. The efficient recruitment of coactivators to the AR involves an association between both the AR N terminus and the LBD, suggesting that substantial folding of the AR occurs to form the transcriptionally competent receptor (2). The CREB-binding protein (CBP), and the orthologue p300 (CBP/p300), form a physical bridging function between the DNA-bound transcrip-
Acetylation Regulates AR Phosphorylation

Acetylation of the tumor suppressor p53 (12), the transcription factors Kruppel-like factor (13), and the erythroid cell differentiation factor GATA-1 (14) enhanced the trans-activation function. More recently, nuclear hormone receptors and nuclear receptor coactivators were shown to serve as targets of acetylation. Direct acetylation of activator of thyroid and retinoic acid receptors ACTR (15) or the ERα (16) contributed to ligand-dependent transcriptional attenuation, whereas direct acetylation of the AR contributed to full ligand-dependent activity (4). Glutamine or threonine substitutions of lysine residues within the AR acetylation site enhanced DHT-dependent activation of androgen-responsive reporter genes, increased physical association with p300, and reduced binding to the N-CoR corepressor (17, 18). Alanine substitution mutants of the AR-acetylated lysine residues showed reduced p300 binding and increased N-CoR binding (17). Together these studies suggested the lysine motif of the AR may serve as an important regulator of coactivator/corepressor binding and acetylation of the AR may be involved in regulating ligand-dependent activity.

Activity of the steroid receptors is also regulated by direct phosphorylation through distinct kinases. The estrogen receptor (ERα) is phosphorylated by the mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) pathways (19, 20) to enhance ligand-independent transcriptional activity. Phosphorylation of the glucocorticoid receptor is induced during S-phase transition (21), and the progesterone receptor phosphorylation is regulated by diverse signals, including the cell-cycle epidermal growth factor and the cAMP pathway (22). In prostate cancer cell lines the phosphorylated AR forms are active, and dephosphorylation of the AR inhibits AR activity (23, 24). Phosphorylation of the 110-kDa AR protein occurs rapidly resulting in the formation of a 110–112-kDa doublet, with a third 114-kDa hyperphosphorylation isoform appearing upon addition of DHT (24, 25). Activation of the cAMP pathway leads to a rapid dephosphorylation of the AR likely through induction of PKA-inducible phosphatases (26). Activity of the AR is enhanced by induction of the MAPK kinase pathway (27, 28).

Post-translational modification by acetylation and phosphorylation under several circumstances may be integrated processes (29–31). Evidence supporting a model that these two post-translational processes may be convergent includes studies of the immediate early (IE) genes such as ternary complex factor and the basal apparatus and contribute to the assembly of high molecular weight “enhancosomes” (reviewed in Ref. 9). p300/CBP convey enzymatic activity toward histones, with the relative activity correlating under certain circumstances with their transcriptional coactivator function. Acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene. Direct acetylation of nonhistone proteins, including transcription factors, regulates AR activity (reviewed in Refs. 10 and 11).

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Post-translational modification by acetylation and phosphorylation under several circumstances may be integrated processes (29–31). Evidence supporting a model that these two post-translational processes may be convergent includes studies of the immediate early (IE) genes such as ternary complex factor (TCF) and histone H3. Contemporaneous with IE gene induction, histone acetylation spanning several nucleosomes is observed. Almost invariably a second nucleosomal modification that occurs upon IE gene induction is the phosphorylation of histone H3 (32) and the nucleosome-binding high mobility group protein HMG-14 (33). Histone H3 is itself both acetylated and phosphorylated, and phosphorylated H3 is more sensitive to the histone deacetylase inhibitor trichostatin A (TSA) than nonphosphorylated H3 (34), providing strong evidence that these two events can be convergent. CREB phosphorylation is augmented indirectly upon activation of the PKA pathway through histone acetylase-regulated alterations in the local chromatin (35, 36). The possibility that nuclear receptors themselves may be the direct target of interdependent acetylation/phosphorylation events remained to be determined.

In previous studies, AR acetylation site alanine substitution mutants conveyed wild type trans-repression of SP-1 and NFκB activity, bound ligand with wild type affinity, and were sumoylated like the wild type receptors. However, induction by the HDAC inhibitor TSA and activation by the p300 coactivator ARS426A, and ARS650A) were described elsewhere (23).

In previous studies suggesting H3 phosphorylation and acetylation may be linked events, we investigated the functional significance of the AR lysine residue motif in signaling by the kinases MAPKK, AKT, and PKA. Mutation of the AR acetylation site did not affect MAPK signaling to the AR. However, the AR acetylation site mutants were defective in regulation by the HDAC inhibitor TSA and by cAMP and AKT signaling. Inhibition of cAMP signaling augmented recruitment of the ARwt to the ARE of the PSA promoter in chromatin immunoprecipitation assays but failed to induce recruitment of the AR acetylation site mutant. Point mutations of six distinct AR phosphorylation sites identified one phosphorylation site regulating HDAC responsiveness. In contrast to the three phosphorylation-dependent isoforms of the wild type AR (110, 112, and 114 kDa), the acetylation mutations lacked the 114-kDa hyper-phosphorylated form, suggesting acetylation and phosphorylation of the AR are functionally convergent events. These studies suggest that the conserved AR lysine residues that are acetylated in vitro and in cultured cells may play a role in coordinating a subset of kinase modules signaling to the AR.

Materials and Methods

Reporter Genes and Expression Vectors—The expression vectors for the protein kinase A catalytic subunit (PKAα), the PKAα mutant (37), and AKT (38) were previously described. Gal4-CREB, and Gal4CREBtrans (39) were previously described. The prostate cancer cell lines DU145 and LNCaP and the promoter reporter plasmids (1.2

Cell Culture, DNA Transfection, and Luciferase Assays—Cell culture, DNA transfection, and luciferase assays were performed as previously described (4, 17, 18). The prostate cancer cell lines DU145 and LNCaP and the HEK293T cell line were cultured in DMEM supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. Cells were plated at a density of 1 × 105 cells in a 24-well plate on the day prior to transfection with LipofectAMINE Plus (Invitrogen). The DNA/LipofectAMINE mixture was added to the cells in Opti-Mem. Cells were incubated in media containing 10% charcoal stripped fetal bovine serum prior to experimentation using dexamethasone (DHT) (4). The 8-bromo-cAMP, forskolin, N-2-p-bromocinnamimylamine-ethyl-5-isoquinolinesulfonamide (H-89), and PD98059 (Calbiochem-Novabiochem International) were reconstituted and stored as recommended by the manufacturer. At least two different plasmid preparations of each construct were used. In cotransfection experiments, a dose response was determined in each experiment with 150 and 300 ng of expression vector and the promoter reporter plasmids (1.2 µg). Luciferase activity was normalized for transfection using β-galactosidase reporters as an internal control. Luciferase assays were performed at room temperature on an Autolumat LB 953 (EG & G Berthold). Cell lysates were determined for 150–300 ng of expression vector with comparison made to the effect of the empty expression vector cassette and statistical analyses was performed using the Mann Whitney U test.

Nuclear Extract Preparation and Western Blots—Preparation of the
cytoplasmic and nuclear extracts from the transfected cells was performed essentially as described previously (40). The antibodies used in Western blot analysis were to the AR (N-20, Upstate Biotechnology, Lake Placid, NY) and to the guanine nucleotide dissociation inhibitor (GDI) (a generous gift from Dr. Perry Bickel, Washington University, St. Louis, MO), which was used as an internal control for protein abundance. For detection of detection of protein, the membrane was incubated with anti-AR (N-20, 1:2000) at room temperature for 2 h. The blots were then washed three times with 0.1% Tween 20 phosphate-buffered saline and incubated with the appropriate horseradish peroxidase-conjugated second antibody. Proteins were visualized by the enhanced chemiluminescence system (Amersham Biosciences). The abundance of immunoreactive protein was quantified by phosphorimaging using an ImageQuant NT densitometry system (Amersham Biosciences).

**Acetylation Regulates AR Phosphorylation**

**RESULTS**

**PKA Inhibition of Liganded AR Function Involves the AR-conserved Lysine Acetylation Motif**—The AR is modified by both phosphorylation and acetylation (4, 17, 18, 23). In recent studies, acetylation and phosphorylation of histones were shown to cooperate in transcriptional activation (29, 43). The androgen-responsive synthetic reporter gene MMTV-LUC was shown to be induced in the presence of ARwt by the specific histone deacetylase inhibitor TSA (4). Using anti-acetyl lysine antibodies the AR was shown to be acetylated in cultured cells, and either p300 or P/CAF acetylated the AR in vitro (4). Edman degradation analysis of the acetylated AR products demonstrated that lysines 630, 632, and 633 were preferentially acetylated. The KLKK sequence constitutes an acetylation motif that is conserved between species (Fig. 1A). The androgen-responsive MMTV-LUC reporter was induced 3-fold at 100 nM DHT (Fig. 1B). We have previously shown that the liganded AR does not induce either the pA3LUC vector or several other luciferase reporter genes (RSV-LUC, cyclin E-LUC, c-fos-LUC), suggesting that the induction of the MMTV-LUC reporter is promoter-specific (44). We investigated the relationship between AR acetylation and phosphorylation function. Activity of the ligand-bound AR is inhibited by PKA stimulators in some (26) but not all studies (45–47), with cell-type, species and reporter-dependent differences described. The isoquinolinesulfonamide H-89 is a specific inhibitor of PKA (48) preferentially inhibiting the PKA (Kᵢ, 50 nm) compared with the PKC pathway (Kᵢ, 76 μM). In our previous studies, H-89 selectively inhibited PKA-induced CAMP-response element (CRE) reporter activity and PKA-induced kinase activity using peptide as substrate, indicating H-89 inhibits PKA signaling both in vitro and in cultured cells (37). Consistent with previous studies (26), inhibition of endogenous PKA activity with H-89 enhanced DHT-induced human AR activity 3-fold in prostate cancer cells (Fig. 1B). In contrast, in the presence of DHT, the ARKwt(632/633A) was induced less than 50%, and the ARK630A mutant was not induced by H-89 (Fig. 1, B and D). The basal activity of both the ARwt and AR mutants was not significantly affected by H-89 (Fig. 1C).

Because these studies suggested PKA-mediated repression of liganded AR activity involve the AR residues acetylated in vitro, we examined the specificity of CAMP-dependent repression of liganded AR activity. Compared with the inactive PKA catalytic subunit mutant or empty expression vector cassette, expression of the PKA catalytic subunit construction induced the activity of the CAMP response element from the glycoprotein a subunit promoter linked to the luciferase reporter gene 15-fold as previously shown (49) (Fig. 2A). Forskolin, an inducer of intracellular CAMP, induced cCRE-LUC activity a further 2-fold compared with empty vector. As predicted, H-89 inhibited PKA-induced CRE activity. The effect on DHT-induced AR activity was assessed using the MMTV-LUC reporter (Fig. 2B). The addition of forskolin reduced DHT-induced AR activity by 40% (p < 0.01) (Fig. 2B, lane 6 versus 8). Forskolin did not affect activity of the ARK630A mutant (Fig. 2B, lane 10 versus 12) but reduced residual activation of the ARK632/633A mutant. An expression vector encoding the catalytic subunit of PKA was transfected into DU145 cells, together with either the AR wt or ARK630Q were transcribed into LNCaP cells using Genejuice transfection reagents (NovaGen). The cells were then treated with vehicle or 100 nM DHT for 24 h, washed with PBS, and fixed in 4% paraformaldehyde for 30 min. Anti-FLAG (rabbit M2, Sigma, 1:100) or anti-PSA monoclonal antibody were applied for detection of FLAG-AR and PSA expression after brief peroxidase-horseradish peroxidase conjugation of the cells using 1% Triton in PBS for 10 min. Secondary antibodies (goat anti-rabbit antibody conjugated with Alexa Fluor 568 for FLAG (catalog #A21069, Molecular Probes, Inc.) and goat anti-mouse antibody conjugated with Alexa Fluor 488 for PSA (catalog #A11017, Molecular Probes, Inc.) were 1:500 diluted in PBS/3% bovine serum albumin and incubated in a humidified chamber for 45 min at 37 °C. The cells were washed with PBS and mounted with Vectashield medium (Vector Laboratory Inc., H-1400).
ARK630A. The 3.5-fold induction of AR activity by DHT was inhibited 30–40% by overexpression of the PKA catalytic subunit (Fig. 2C). In contrast, the liganded AR_{K630A} was not repressed by either the expression of the PKA catalytic subunit or the addition of forskolin (Fig. 2, B and D). To examine further the specificity of cyclic AMP signaling in DU145 cells, a heterologous reporter system was used (Fig. 2E). The CREB transcription factor linked to the Gal4 binding domain was assessed using the Gal4 DNA binding sites linked to a luciferase reporter gene. Consistent with studies performed on other cell types, coexpression of the catalytic subunit of protein kinase A activated transcriptional activity of CREB ~8-fold (Fig. 2F).
**Fig. 2.** cAMP repression of AR wt but not the AR acetylation site mutants. 

A, the canonical cAMP response element (CRE) was assessed for activity in DU145 cells in the presence of coexpressed PKA wild type or PKA mutant expression plasmid. Alternatively, cells were treated with the cAMP activator forskolin (20 μM) for 24 h (left panel). The induction of α-CRE-LUC reporter activity is shown. The right panel shows the inhibition of cAMP-induced activity of the α-CRE reporter by H-89 addition.

B, the MMTV-LUC reporter was transfected into DU145 cells together with an expression vector for either the PKA catalytic subunit or the inactive PKA catalytic subunit mutant and either the ARwt, or the ARK630A or ARK632/633A mutant. DHT treatment was for 24 h. The data are the mean ± S.E. of six separate transfections. C and D, DHT-induced activity...
The mutant of protein kinase A catalytic subunit reduced basal CREB activity ~50%. Point mutation of the protein kinase A phosphorisation site of CREB at serine 133 abrogated protein kinase A catalytic subunit-dependent activation of CREB. This point mutation was also defective in repression by the PKA catalytic subunit mutant. These studies demonstrate that the PKA catalytic subunit induces protein kinase A signaling in a specific manner in DU145 cells and does not have an independent effect on luciferase reporter gene activity. Together these studies suggest PKA expression or forskolin treatment activates cAMP signaling in DU145 cells and inhibits DHT-induced activity of the AR, but they do not affect the activity of the liganded ARK630A or ARK632/633A mutants.

The AR Acetylation Site Regulates AR Activity Induced by JNK but Not Endogenous MAPK in Prostate Cancer Cells—Recent studies demonstrated the AR acetylation site was required for MEKK1-mediated apoptosis in DU145 cells (17). To determine whether the AR acetylation site affects AR signaling by other serine threonine kinase pathways, experiments were conducted with regulators of the ERK mitogen activated protein kinase (MAPK) pathways. The c-fos promoter linked to a luciferase reporter gene was induced by coexpression of either MAPKK or the constitutively active MAPKK mutant (RΔF) 8- to 9-fold. The catalytically dead mutant of MAPKK (K97M) failed to induce c-fos reporter activity as previously described (50). The addition of PD98059 reduced MAPKK-dependent induction of c-fos by 40% at 5 μM (Fig. 3A), indicating that PD98059 was capable of inhibiting ERK-dependent reporter gene activity in DU145 cells. We next examined regulation of the MAPK-responsive AP-1-responsive reporter gene, p3TP-LUX. The constitutively active MAPKK induced AP-1 reporter activity 9-fold (Fig. 3B). The catalytically dead mutant of MAPKK (K97M) failed to induce AP-1 reporter activity. PD98059 inhibited MAPKK-induced AP-1 reporter activity by 70% indicating the MAPK pathway inhibitor PD98059 (5 μM) was effective in DU145 cells (Fig. 3B). The RΔP mutant induced MMTV-LUC activity 2–5 fold and further enhanced AR activity in the presence of DHT (Fig. 3C). The activity of ARK630A was induced by expression of RΔF 2-fold in the absence and 3-fold in the presence of DHT. ARK632/633A was induced 2-fold in the absence and 4-fold in the presence of DHT. The ~2-fold induction or activation by the MAPK pathway of the AR acetylation mutants was preserved. Because activation of MAPK signaling pathway in DU145 cells induced activity of the AR acetylation mutant, further analysis was conducted to determine the specificity of MAPK-dependent induction of gene transcription in DU145 cells. Analysis was conducted of reporter genes encoding a region of the c-fos promoter within ~355 and ~279 that includes the SIE (cis-inducible element), TCF, and SRE site of the c-fos promoter. Previous studies have demonstrated the induction of c-fos promoter activity by epidermal growth factor required the MAPK signaling pathway in the TCF site. The c-fos promoter construct was induced 7-fold by the activating MAPK kinase expression vector but was not induced by the point mutant MEK672TM (Fig. 3D). In contrast, mutation of the SRE site reduced MAPK kinase-dependent induction of c-fos reporter activity to ~5-fold and mutation of the TCF site abrogated MAPK kinase-dependent induction of c-fos activity in DU145 cells. Together these studies demonstrate DNA sequence-dependent activation of reporter gene activity in DU145 cells by MAPK kinase.

AR activity is inhibited by activation of the AKT pathway and phosphorylation of the AR is required for AKT-repression (51). To examine the functional significance of the AR acetylation site in ARK-induced prostate cellular apoptosis, studies were performed in DU145 cells as previously described (52). Cells were cotransfected with wild type or mutant ARs, the AKT expression vector, or control empty vector. Cells were treated for 24 h with either DHT (10−7 M) or control vehicle. Coexpression of AKT and the ARwt in the presence of ligand reduced AR activity ~50% as previously described (53). Coexpression of AKT with either ARK630A or ARK632/633A had no significant effect on the activity of the mutant AR compared with vector controls (Fig. 4A). Similar observations were made using the androgen-responsive PSA-LUC reporter (Fig. 4B). To examine further the specificity of AKT signaling in DU145 cells, the c-fos-responsive promoter was again employed. Overexpression of AKT induced c-fos activity 2-fold. This activity was inhibited by the phosphatidylinisolosin 3-kinase inhibitor LY294002 ~50%. Coexpression of the dominant negative mutant of AKTK179M reduced basal c-fos promoter activity consistent with the presence of basal AKT activity in DU145 cells. Together these studies demonstrate an important function of the AR acetylation site in regulating AKT-dependent signaling in cultured human prostate cancer cells.

Experiments were conducted to determine the functional consequence of the AR acetylation site in cultured cells. Initial experiments were conducted comparing the effect of the AR acetylation mutant on the wild type androgen receptor. The wild type androgen receptor induced the PSA promoter ~5-fold in the presence of DHT. Coexpression of the AR acetylation mutant (ARK630A) reduced AR-dependent induction of the PSA promoter ~40% in equal molar amounts (Fig. 5A). The AR LNCaP mutant induced PSA activity ~2-fold. Coexpression of the AR acetylation site mutant ARK630A abrogated DHT-induced activation of PSA by the LNCaP AR mutant (Fig. 5B). Comparison was next made of the AR acetylation site on abundance of the PSA protein in LNCaP cells (Fig. 5C). ARK630A was made between the ARwt, the ARK630A, ARK632/633A, and an acetylation mimic mutant of the acetylation site ARK360Q. Immunohistochemical staining was conducted for either the FLAG epitope or the coexpressed androgen receptors or PSA. Coexpression of the acetylation mimic mutant increased PSA abundance within cells containing the activating mutant receptor. The addition of DHT further enhanced the induction of PSA in the presence of either the ARwt or ARK360Q. However, cells coexpressing the ARK630A or ARK632/633A demonstrated significantly reduced PSA immunostaining. Together these studies suggest the acetylation site mutant functions as a dominant negative inhibitor of androgen receptor signaling.

The AR Acetylation Site Mutation Affects the Isoelectric Focus of the AR in Both the Liganded and Unliganded State—The ARwt and acetylation site mutants bound ligand with similar affinity by Scatchard analysis and gave similar gross structural analysis by tryptic digestion (17). The current studies suggested a role for the acetylation site in regulating ligand-induced covalent modifications (e.g. phosphorylation and acetylation) in the receptor. In previous studies, non-denaturing isoelectric focusing in ultra-thin polyacrylamide gels with subsequent two-dimensional electrophoresis was used to assess conformation of the liganded human AR (54). To assess the effect of mutation within the AR acetylation site on covalent
Induction of AR activity by MAPK does not require the AR acetylation site. A, the native c-fos promoter linked to a luciferase reporter gene was induced by coexpression of either MAPK kinase-activating mutant (MEK<sub>R</sub>AF) or MEK<sub>wt</sub> but not the kinase-dead mutant (K97M). B, PD98059 inhibition of ERK-dependent reporter activity was assessed using the AP-1-responsive reporter gene, p3TPLUX and co-expressed MAPK kinase-activating mutant (MEK<sub>R</sub>AF). C, the androgen-responsive reporter MMTV-LUC was used to assess AR-dependent MAPK signaling. Cells transfected with either AR<sub>wt</sub> or AR mutants with or without MAPK kinase-activating mutant (MEK<sub>R</sub>AF) were treated with DHT or vehicle for 24 h. D–F, the c-fos promoter luciferase reporter with point mutations in the sis-inducible element, TCF, or SRE site as indicated were assessed for induction by the MAPK kinase expression vectors. Data are mean ± S.E.
modification of the AR, the ARwt and AR_{K630A} were expressed in HEK293 cells and treated with either vehicle or DHT for 24 h. The AR was immunoprecipitated with an hAR antibody and the immunoprecipitated AR was subjected to two-dimensional gel electrophoresis. In the absence of ligand, the ARwt migrated as a protein of ~110 kDa and focused as two groups of species, with three dominant protein spots of pH 4.5 and two major spots at pH 6 (Fig. 6A). Additional minor spots spread down to around pH 5 (Fig. 6B) consistent with previous studies (54). In the presence of ligand, the ARwt focused at one major spot at pH 5.2 with spots of less protein distributed at pH 4.5, 4.8, 5.5, and 6 (Fig. 6B). In the absence of ligand, the AR_{K630A} mutant showed three spots around pH 4.5 with migration at pH 6 (Fig. 6B), consistent with previous observations (4, 17). Upon DHT treatment, the AR_{K630A} mutant migrates as a major spot at pH 5.2 and a minor spot at pH 4.8. Spots within other pH range seen in the ARwt in the presence of ligand were not detectable (Fig. 6B). In the presence of ligand, the ARwt focused at one major spot at pH 5.2 with spots of less protein distributed at pH 4.5, 4.8, 5.5, and 6 (Fig. 6B). In the absence of ligand, the AR_{K630A} mutant showed three spots around pH 4.5 with migration at pH 6 (Fig. 6B), consistent with previous observations (4, 17). Upon DHT treatment, the AR_{K630A} mutant migrates as a major spot at pH 5.2 and a minor spot at pH 4.8. Spots within other pH range seen in the ARwt in the presence of ligand were not detectable (Fig. 6B) even with substantially longer exposures (data not shown). The experiments were conducted on multiple occasions with the same findings. These results suggest that mutation of the AR acetylation site affects its covalent modifications; leading to changes in the isoelectric profile of the AR. Substitution of alanine for lysine (AR_{K630A}) would be anticipated to reduce positive charge and pI (isoelectric point) as observed. The addition of ligand induced multiple ARwt species which were not observed with the AR_{K630A}. Suggesting several covalent modifications are dependent upon the lysine residue.

The AR Acetylation Site Regulates Formation of the 114-kDa Form of the AR—The AR is synthesized as a nonphosphorylated protein and migrates as a 110-kDa protein during SDS-PAGE. The AR becomes phosphorylated at serines within 15 min after synthesis and then migrates as a doublet of 110–112 kDa. In response to ligand, a third (114 kDa) isoform is induced (25) and, in the presence of protein kinase A stimulators (cAMP or forskolin), the AR is rapidly dephosphorylated (26). This has been observed (25) and, in the absence of protein kinase A stimulators (cAMP or forskolin), the AR is rapidly dephosphorylated (26). To assess the electrophoretic properties of the ARwt and AR_{K630A} mutant, HEK293T cells were transfected with the AR expression vectors and cellular extracts assessed by SDS-PAGE. In DHT-treated cells, the three AR isoforms (110–114 kDa) were observed (Fig. 7, A and B, a–c). The incubation of extracts with calf intestinal alkaline phosphatase abolished the presence of 112- and 114-kDa bands (Fig. 7A, lane 2 versus lane 1). The ARwt and AR acetylation mutant were transfected into HEK293T cells, treated with DHT for 24 h, and subjected to Western blotting. In contrast with the ARwt, the AR_{K630A} or AR_{K632/633A} mutants did not display the 114-kDa isoform (Fig. 7B), suggesting ligand-induced phosphorylation requires the acetylation site. Multiple experiments of Western blotting of transfected cells demonstrated similar levels of expression of the ARwt and mutants receptors in the basal and the DHT-treated state (data not shown) as previously described (4, 17). These studies suggested acetylation may be required for the AR phosphorylation and formation of the higher molecular weight forms.

The subcellular localization of the nuclear receptors is regulated by the ligand. The DNA-binding domain and the flanking hinge regions harbor a bi- or tripartite-type nuclear localization signal (NLS), consisting of two or three clusters of lysine and arginine residues (5, 55). The AR contains a second NLS embedded in the ligand-binding domain, similar to ERα and progesterone receptor (55). The acetylation motif (KLKK) of the AR in the hinge region constitute the core of the first NLS (NL1) of the AR. To examine if the acetylation site mutant would affect the nuclear trafficking of the AR, the DNA constructs expressing either ARwt or AR_{K632/633A} mutant were transfected into HEK293T cells. The cells were then treated with or without 100 nM DHT for 24 h. The cytoplasmic/nuclear cellular lysates of the cells were extracted and subjected to SDS-PAGE and Western blotting with anti-AR antibody. DHT treatment enhanced nuclear transfer of the ARwt. The AR_{K632/633A} mutant was also detectable in the nuclear fractions after DHT treatment (Fig. 7C), consistent with previous observations that deletion of the AR acetylation motif (AR_{K629–633}) results in delayed but none-the-less complete ligand-dependent
The current studies suggest the AR acetylation site regulates both ligand-induced and H89-induced AR signaling. To examine further the evidence for cross-talk between acetylation and phosphorylation at the level of the androgen receptor, chromatin immunoprecipitation assays were conducted to assess whether PKA signaling may regulate androgen receptor occupancy at an ARE in the context of its local chromatin structure. The ARE of the PSA promoter was used for ChIP assays. HEK293T cells were transfected with either FLAG-tagged ARwt, the AR acetylation site mutant (AR K630A, ARK(632/633)A), or the AR acetylation site mimic mutant (AR K630Q). Cells were treated with vehicle or DHT for 24 h, and immunohistochemical staining is shown for FLAG-tagged AR and PSA proteins.
AR<sub>wt</sub> (Fig. 8A) or FLAG-tagged AR<sub>K630A</sub> (Fig. 8B). DHT induced recruitment of the androgen receptor to the ARE, consistent with the induction of ARE signaling by the HDAC inhibitor TSA. Furthermore, the PKA inhibitor H89 induced recruitment of AR to the ARE. The AR acetylation site mutant demonstrated substantially reduced recruitment to the PSA promoter in the presence of DHT. The ARK630A mutant demonstrated substantially reduced recruitment to the PSA promoter in the presence of DHT. The relative induction of the ARK630A mutant to the PSA promoter by TSA was also substantially reduced. H89 treatment of cells resulted in no detectable recruitment of the AR acetylation site mutant to the PSA promoter. Although the relative abundance of the AR wild type or AR acetylation site mutant in nuclear extracts in the presence of DHT was similar (Fig. 7C), the recruitment to an ARE in the context of local chromatin assessed using ChIP assays was substantially reduced. These studies suggested that protein kinase A-dependent regulation of AR recruitment to an ARE was abrogated by the acetylation site mutation.

The defective recruitment of the AR acetylation site mutant to an ARE in ChIP assays suggested evidence for cross-talk between phosphorylation and acetylation of the androgen receptor. To determine whether the AR phosphorylation in turn affected acetylation-dependent functions of the AR, a series of point mutation substitutions of the androgen receptor were assessed (23). Comparison was made among mutants of the androgen receptor at the phosphorylation sites where the phosphorylated serine residues were substituted with alanine (Fig. 9A). The androgen-responsive reporter gene PSA luciferase was examined, and a comparison was made between the androgen receptor wild type and the phosphorylation site mutants (Fig. 9B). Consistent with the previous publication (23), each of the phosphorylation site mutants induced androgen signaling ~5-fold. The S308A mutant enhanced DHT-dependent trans-activation significantly more than AR wild type as previously described in LNCaP cells (23).

To assess the role of HDAC-dependent signaling to the phosphorylation site mutants, the specific HDAC inhibitor TSA was
used. Regulation of AR signaling was selectively reduced by the S94A mutant. Induction by TSA was reduced ~50% (Fig. 9C). In previous studies of the p300-dependent trans-activation of AR signaling, the HAT domain was required, and the acetylation site mutants were defective in p300-dependent trans-activation (4). The phosphorylation site mutants were therefore examined for trans-activation by p300. The S94A mutant was significantly reduced in trans-activation by p300 (Fig. 9D).

Western blot analysis was conducted of the AR phosphorylation site mutants, and normalization for protein transfer was conducted using the control GDI. The relative abundance of the phosphorylation site mutants was similar when normalized to the loading control, other than the S424A, which showed ~50% reduction in protein abundance in the presence of DHT. Together these studies demonstrate that the AR phosphorylation site residue Ser94 plays an important role in activation by the p300 histone acetyltransferase and regulation by the histone deacetylase inhibitor TSA.

DISCUSSION

The current studies demonstrate the conserved AR acetylation site plays an important role in a subset of kinase signaling pathways previously shown to regulate AR activity. AR acetylation-defective mutants showed reduced regulation by AKT, PKA, and JNK, whereas MAPK and sumoylation of the AR were unaffected by point mutation within this acetylation site. Together with previous studies demonstrating this site does not affect AR trans-repression of NFκB or SP-1 activity (11, 18), the current findings suggest post-translational modification by acetylation may coordinate a distinct subset of AR functions. Growing evidence suggests a model in which acetylation and phosphorylation of histones contribute to a local signaling module (31, 56–58). Post-translational modification within the histone tails in turn contribute, through changed charge, to recruitment of other histone acetyltransferase or chromatin modeling proteins. In view of the recent studies that AR acetylation mutants substituting lysine for arginine or alanine are defective in binding the histone acetyltransferase p300 and show enhanced recruitment of nuclear N-CoR-Smad-HDAC-1 complexes (17), the current studies suggest transcription factor acetylation sites may also coordinate recruitment of HAT, and

Fig. 7. Phosphate incorporation into the AR involves the AR acetylation site. A, the ARwt was transfected into HEK293T cells, and cell extracts harvested after 24 h of DHT treatment were either untreated or treated with calf intestinal alkaline phosphatase and subjected to SDS-PAGE electrophoresis. Western blotting is shown for the AR. The AR shows the presence of three isoforms (a, b, and c). Treatment with calf intestinal alkaline phosphatase abrogates c. B, the ARwt, ARK(632/633)A, and ARK630A mutant were transfected into HEK293T cells and treated with DHT for 24 h. Cell extracts were harvested, and Western blot analysis was performed for the AR. The acetylation site mutants do not display isoform c shown in A, lane 1. C, expression vector of ARwt and ARK(632/633)A mutants were transfected into HEK293T and treated with DHT for 24 h. The cytoplasmic and nuclear lysates were extracted and separated on 7% SDS-PAGE gel and blotted with anti-AR antibody.

Fig. 8. AR recruitment to the ARE of PSA promoter. Chromatin immunoprecipitation (ChIP) assays were conducted in HEK293T cells transfected with either FLAG-ARwt (A) or FLAG-ARK630A (B). Cells were treated with DHT (10 nM), H89 (50 nM), or TSA (100 nM) and ChIP assays conducted with antibodies as indicated. Note the 1X and 10X exposure of the PCR product of anti-FLAG IP to detect FLAG-tagged ARwt and ARK630A.
serve as signaling modules, including phosphorylation.

The AR point mutants substituting lysine for alanine within the acetylation motif were defective in activation by coactivators (p300, SRC-1a, Ubc-9, and TIP60) and bound relatively more N-CoR suggesting a critical role for direct receptor acetylation in corepressor and coactivator engagement (17). How might acetylation within the AR hinge region affect H-89-dependent activation of the AR? The AR acetylation site does not fall within the ligand-binding pocket deduced from the crystal structure (59), consistent with the wild-type ligand binding of the AR acetylation site mutants. Expression levels of the ARwt, the ARK630A, or ARK(632/633)A mutant proteins were also similar in cultured cells (4, 17). DHT induces AR phosphorylation (24) and forskolin-induced dephosphorylation of the AR in prostate cancer cells may involve PKA-inducible phosphatases such as nuclear protein phosphatase-1 (26). Analysis of Ser641, one of the two serine residues dephosphorylated in response to cAMP (serines 641 and 653) (26), demonstrated the importance of Ser641 to the overall phosphorylation of the AR. Ser641 is located in the AR hinge region in close proximity to the KXXK motif. One interpretation of the finding that the AR acetylation site mutants failed herein to form the 114-kDa isoform and were resistant to PKA repression is that ligand-induced coactivator recruitment, dependent upon the AR lysine residues, precedes phosphorylation of the AR. Because DHT-induced accumulation of AR in the nucleus occurs rapidly, within minutes, and appears to precede receptor phosphorylation, it has been proposed that AR phosphorylation may play a role in late

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**Fig. 9.** Regulation of AR phosphorylation site mutants by the histone deacetylase inhibitor TSA and the histone acetyl transferase p300. A, schematic representation of the AR phosphorylation site. B–D, the expression vectors encoding the AR phosphorylation site mutants were expressed in HEK293T cells and analyzed for regulation by DHT, TSA, or p300 trans-activation. The data are mean ± S.E. for n ≥ 6 separate transfections. E and F, Western blot analysis of HEK293T cells transfected with the expression vector of the phosphorylation site mutants was conducted with antibodies to the AR. GDI is control for protein abundance and transfers.
phases of transcriptional regulation or receptor recycling (23). The failure of the liganded ARK360A mutant to form several species observed with the ARwt by two-dimensional electrophoresis is also consistent with the role of the lysine residues in subsequent ligand-induced conformational modifications.

In the current studies, chromatin immunoprecipitation analysis demonstrated ligand-induced recruitment of the AR wild type to the androgen response element of the PSA promoter in response to the ligand DHT, the histone deacetylase inhibitor TSA, or the protein kinase inhibitor I89. The recruitment of the AR acetylation site mutant was dramatically reduced despite similar levels of mutant receptor in nuclear extracts. The defective recruitment of the AR acetylation site mutant is consistent with recent studies of p53 acetylation (80). These findings are also consistent with previous studies in which DHT recruited AR to the PSA promoter. TSA treatment hyperacetylated histone H3 at lysine 9 (34) and hyperacetylation of H3 lysine 9 is thought to correlate with transcriptional activation of target genes by altering chromatin structure and enhancing transcription factor recruitment to target DNA sequences. The PKA inhibitor I89 enhanced AR recruitment to an ARE, an effect abrogated upon mutation of the AR acetylation site lysine motif. How might PKA affect AR recruitment? PKA is known to induce phosphorylation of histone H3 at lysine 10 (29), which may in turn alter local chromatin structure to enhance transcription factor access. Such a possibility is supported by the finding that phosphorylation of histone H3 increases the susceptibility of histone H3 to HATs or HDAC inhibitor to induce histone H3 acetylation (29, 43). Alternatively, recent evidence for cross-talk between PKA signaling and histone hyperacetylation is a finding that PP1 physically associates with HDAC, contributing to regulation of CREB activity (61). The possibility that AR-bound HDAC could recruit PP1 to regulate AR signaling remains to be determined.

Additional evidence for interdependence of phosphorylation and acetylation was derived from analysis of AR phosphorylation site mutants. TSA induction of AR signaling was maintained for each of the AR phosphorylation site mutants except substitution of serine 94. Similarly, induction of AR signaling by the histone acetyltransferase p300 was selectively reduced by this mutation of the phosphorylation site. Further mechanistic insight into the relationship between AR acetylation and phosphorylation will require a better understanding of how AR phosphorylation regulates the diverse functions of the AR.

The AR acetylation mutants ARK360A or ARK363A did not form the 114-kDa phosphorylated isofrom observed with the ARwt in the presence of ligand. Several recent observations suggest acetylation and phosphorylation of histones may function as integrated post-translational modifications and in some circumstances can cooperate to activate transcription (29, 43, 62). Histone H3 phosphorylation at Ser10 enhances acetylation at Lys14 (43). The functional cooperation between phosphorylation and acetylation may involve a phosphate-dependent stabilization of the enzyme substrate complex. Thus the histone acetyltransferase Gcn5 displays a 10-fold greater preference for phosphorylated H3 over nonphosphorylated H3 (43). Cooperation between phosphorylation-acetylation cascades of transcription factors had been shown for p53 function in response to DNA damage (31, 63). Although in vitro studies have shown phosphorylation of Positive Coactivator 4 by caseine kinase II inhibits acetylation by p300 (64) and an inverse correlation between phosphorylation and acetylation of the forkhead transcription factor has been observed (65), further analysis of these proteins will require identification of specific residues and mutational analyses.

In the current studies, mutation of the AR lysine residues did not affect ERK signaling to the AR. These findings are consistent with analyses of histone H3 lysine acetylation, which is also independent of MAPK (34). Although several studies have implicated direct transcription factor acetylation in regulating reporter gene expression, only recently has the functional cellular phenotype governed by transcription factor acetylation been examined (66). The AR is overexpressed or promiscuously activated in human prostate cancer, and the effectiveness of androgen ablation therapy in reducing prostate cancer cellular growth suggests a key role for the liganded AR in aberrant prostate cell growth (67). AR acetylation site lysine residue substitutions with glutamine to mimic charge changes induced by lysine acetylation promote contact-independent growth of prostate cancer cell lines in soft agar and in nude mice and resistance to flutamide. Furthermore, AR acetylation mimic mutants selectively enhance activity of the cell-cycle control genes cyclin D1 and cyclin E (18). Acetylation site mutations of p53 were defective in repression of Ras-induced transformation, suggesting an important functional role for p53 acetylation in vivo (66). The identification of acetylation as a post-translational modification required for regulation of the AR by a distinct subset of signaling pathways (AKT, PKA, and JNK) and not MAPK or sumoylation suggests these residues contribute to a new type of signaling specificity.

Post-translational modification of histones contributes to a signaling “code” and forms a platform for recruitment of distinct proteins. Lysine residues of histones, which can be either acetylated or methylated, result in a mutually exclusive modification. The in vitro binding of TAF1 to50 to multiply-acetylated H-4 tails (68) suggests pairs of acetylated residues with particular spacing recruit specific bromo-domain containing proteins. In the same manner that combinatorial modifications, including phosphorylation and acetylation, form signaling platforms recruiting proteins to histones, these modifications appear to encode signaling platforms for nuclear proteins. The recruitment of candidate proteins to lysine residues of nuclear receptors may result in steric hindrance and thereby exclude the binding of other kinases that regulate AR signaling.

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