Ligand Binding to the Androgen Receptor Induces Conformational Changes That Regulate Phosphatase Interactions[▽]

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We describe a mechanism for protein phosphatase 2A (PP2A) targeting to the androgen receptor (AR) and provide insight into the more general issue of kinase and phosphatase interactions with AR. Simian virus 40 (SV40) small t antigen (ST) binding to N-terminal HEAT repeats in the PP2A A subunit induces structural changes transduced to C-terminal HEAT repeats. This enables the C-terminal HEAT repeats in the PP2A A subunit, including HEAT repeat 13, to discriminate between androgen- and androgen antagonist-induced AR conformations. The PP2A-AR interaction was used to show that an AR mutant in prostate cancer cells (T877A) is activated by multiple ligands without acquiring the same conformation as that induced by androgen. The correlation between androgen binding to AR and increased phosphorylation of the activation function 1 (AF-1) region implies that changes in AR conformation or chaperone composition are causal to kinase access to phosphorylation sites. However, AF-1 phosphorylation sites are kinase accessible prior to androgen binding. This suggests that androgens can enhance the phosphorylation state of AR either by negatively regulating the ability of the ligand-binding domain to bind phosphatases or by inducing an AR conformation that is resistant to phosphatase action. SV40 ST subverts this mechanism by promoting the direct transfer of PP2A onto androgen-bound AR, resulting in multisite dephosphorylation.

The nuclear receptor superfamily of transcription factors directs the expression of genes whose products regulate diverse biological pathways. The domain organization of nuclear receptors is conserved and includes an N-terminal activation function 1 (AF-1) region, a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD). Ligand binding to the LBD initiates a series of changes in nuclear receptor structure, chaperone composition, localization, trans-activation potential, and protein half-life ($t_{1/2}$) (10, 28, 36, 44). Understanding how ligand binding elicits these changes is fundamental to understanding nuclear receptor regulation and activity. Defining how ligands control nuclear receptor activity should also provide insight into certain disease mechanisms and aid in the design of drugs such as selective androgen receptor modulators and selective estrogen receptor modulators (4, 27).

In addition to ligand binding, nuclear receptors can be regulated by signal transduction pathways. Kinases including those controlled by growth factor-dependent pathways act directly or indirectly on a variety of nuclear receptors (37). Kinases reported to regulate androgen receptor (AR)-dependent transcription include the mitogen-activated protein kinases (p42/44, p38, and Jun N-terminal protein kinase), protein kinase A, and protein kinase C (8). The mitogen-activated protein kinases p38 and Jun N-terminal protein kinase also regulate the nucleocytoplasmic distribution of AR (15). Determining exactly how kinases regulate nuclear receptor transcription activity has

been challenging because of cross talk between signaling pathways and because gene expression pathways contain numerous potential kinase targets (25). Perhaps the best-characterized example in this regard is phospho-regulation of the estrogen receptor (ER). Direct phosphorylation of ER provides the basis for an estrogen-independent pathway that promotes co-activator recruitment (18, 41).

Information addressing how dephosphorylation contributes to nuclear receptor regulation is limited. Experiments with the black sponge-derived toxin okadaic acid (OA) have implicated phosphatases in the control of glucocorticoid receptor (GR) localization and activity (11, 39). The phosphatases targeted by OA in these experiments include protein phosphatase 2A (PP2A) and PP5. Additionally, it has been shown that knock-down of PP5 protein levels promotes the cytoplasmic distribution of GR (9), suggesting that nuclear transport of GR is regulated by PP5. Despite these and other intriguing observations linking phosphatases to nuclear receptor function, evidence that a phosphatase directly dephosphorylates a nuclear receptor and that dephosphorylation is causal to a change in transcription activity has not been unequivocally demonstrated.

PP2A is one of the most abundant phosphatases in mammalian cells. The PP2A core enzyme is a heterodimer that contains a catalytic C subunit and a structural A subunit. The PP2A A/C heterodimer is targeted to protein substrates by a third subunit known as a B subunit (21). We recently reported that phosphatase PP2A is a component of AR complexes isolated from simian virus 40 (SV40)-transformed cells (48). Unexpectedly, PP2A binding to AR occurs through an atypical substrate targeting reaction in that it does not involve a PP2A B subunit. Rather, PP2A targeting in this system relies on

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A-subunit contact with AR, an interaction that is induced by SV40 small t antigen (ST). ST is known to bind directly to HEAT (for *Huntington*, elongation factor, *A* subunit, *TOR*) repeats (1) in the A subunit, and ST binding causes displacement of B subunits and reduces PP2A catalytic activity (32, 35). PP2A, once transferred from ST to AR, is catalytically active. Phospho-site-specific antibodies were used to show that PP2A dephosphorylates five phosphoserines in the AF-1 region of AR (48). ST expression reduces AR transactivation of luciferase-based reporter genes, but it increases ER-dependent transcription (48). These data suggested that either AR or a component of the AR transcription complex is a PP2A substrate that, when dephosphorylated, results in a reduction of transcription.

In the present study we describe the mechanism of ST-dependent PP2A transfer onto AR. We present evidence that ST binding induces a conformational change that is transduced ~ 76 Å through tandem HEAT repeats in the PP2A A subunit. The conformational changes generate an AR binding site in C-terminal HEAT repeats in the PP2A A subunit. A remarkable feature of the ST-induced conformational change in the PP2A A subunit is that it discriminates between different ligand-bound forms of AR. Thus, the A subunit can function as a targeting subunit for the PP2A A/C heterodimer, and HEAT repeats in the PP2A A subunit can adopt a structure that is capable of recognizing relatively subtle differences in substrate structure. Recent crystallographic analysis of PP2A has revealed a flexible region in the C terminus that includes HEAT repeats 13 to 15 (5, 17, 46, 47). We found that deletion of HEAT repeat 13 is sufficient to abolish binding to AR. In the course of analyzing the AR domains that mediate PP2A interactions, we made several observations that address the more general question of how ligand binding increases the level of nuclear receptor phosphorylation. It has generally been assumed that ligand binding to nuclear receptors increases phospho-site availability to kinases. We show, however, that kinases can access phospho-sites in AR prior to androgen addition. Phosphatase access and/or targeting to AR was found to be dependent on the LBD. Our data suggest that ligand binding increases the phosphorylation state of multiple AF-1 phospho-sites by repressing the action of phosphatases on AR. We propose that phosphatase action is a key determinant of AR phosphorylation state.

MATERIALS AND METHODS

Reagents. AR antibodies, epitopes, and sources are as follows. Monoclonal antibody (MAb) AR441 is an anti-AR mouse MAb generated against residues 299 to 315 (STEDTAEYSPFKGGYTK) (30) and was a kind gift from D. Edwards. G122-434 is an anti-AR mouse MAb against AR generated against residues 33 to 485 and was purchased from BD Pharmingen. AR441 and/or G122-434 was used to isolate AR complexes by immunoprecipitation (IP). Two rabbit polyclonal antibodies to AR were prepared, affinity purified by standard methods, and used for immunoblotting. AR21 was generated against residues 1 to 21 (MEVOLGLGRVYPRPPSKTYRG), and the anti-AR hinge region was generated against residues 656 to 669 (TQKLTVSHIEGYEC). AR phospho-site antibodies (pS in the sequences indicates phosphoserine) used were pSer16 (CYPRPPpSKTYRG), pSer81 (Upstate), pSer94 (CQGEDGpSPQAH), pSer256 (CALEHLpSPGEQL), pSer308 (CDTAEYpSPFKGG), and pSer424 (CGPGSGpSPSAAA) (48).

Other antibodies used and their sources are as follows. PAb108 (American Type Culture Collection) is a mouse MAb against SV40 ST that also cross-reacts with Jamestown Canyon virus (JCV) ST and was used in immunoblotting. PAb430 is a mouse MAb that recognizes native SV40 ST and was used to immunoprecipitate SV40 ST-PP2A complexes. PP2A C-subunit antibodies used

include a mouse monoclonal 1D6 (Upstate) and a rabbit polyclonal antibody (29). PP2A A-subunit antibodies used include a goat polyclonal antibody (SC-6112; Santa Cruz) and a rat monoclonal antibody (MRT-204R; Covance). The rabbit polyclonal antibody against the C subunit and the goat polyclonal antibody against the A subunit were used in immunoblotting analyses; all antibodies against the PP2A A or C subunit were tested in electrophoretic mobility shift assays (EMSAs). MU014-UC is a rabbit polyclonal antibody against prostate-specific antigen (PSA; BioGenex). DM1A is a mouse MAb against tubulin (Sigma). 16B12 is a mouse MAb against the hemagglutinin (HA) epitope (Covance). M2 is a mouse MAb against the Flag epitope (Sigma).

Transfection reagents used were Eugene6 (Roche) for PC-3, Cos7, 293, and 293T cells and Transfectin (Bio-Rad) for PC-3 and LNCaP cells. The AR ligands used were 5-dihydrotestosterone (DHT), androstenedione (ASD), dehydroepiandrosterone (DHEA), estradiol, flutamide (Flut), and hydroxylflutamide (HO-Flut), all from Sigma; synthetic androgen methyltrienolone (R1881) and the radiolabeled form [3 H]R1881 from Perkin-Elmer; and bicalutamide (Casodex) from Fisher Scientific. Immunoblotting was performed with peroxidase-labeled secondary antibodies by enhanced chemiluminescence (Pierce). Optimal film exposures were scanned, normalized to a blotting control, and quantified using ImageJ.

IP assays. Cells were suspended in 5 volumes of Triton lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a 5 μ g/ml of aprotinin, leupeptin, and pepstatin A) and incubated on ice for 20 min with occasional mixing. The lysates were clarified by centrifugation (18,000 $\times$ g for 15 min), and the supernatants were rotated with antibody-protein G beads (4 μ g of antibody/10 μ l of packed beads) at 4°C for 4 h. After five washes with Triton lysis buffer, AR complexes were either eluted by incubating the beads with synthetic peptide (20 μ g/ml) at room temperature for 60 min or by resuspending the beads in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95°C. AR complexes were separated by SDS-PAGE followed by Western blot analysis and/or silver staining by standard methods. Immunoblot analysis of PP2A subunits that coimmunoprecipitate with AR was performed with antibodies to the A subunit, the C subunit, or both. Because of the stability of the A/C heterodimer, C-subunit detection is a reliable indicator of the presence of the A subunit.

Androgen binding assays in vivo. PC-3 cells were grown in RPMI phenol red-free (PRF) medium containing 5% fetal bovine serum (pretreated with charcoal and dextran). The cells were infected with adenovirus (Ad)-HisAR, an Ad encoding human AR with a deletion of residues 1 to 37 and an N-terminal His₆ tag (kindly provided by William Walker) and with Ad-ST, an Ad encoding SV40 ST (kindly provided by K. Rundell) (33). The infected cells were washed three times with phosphate-buffered saline (PBS) and then incubated with labeled synthetic androgen (0.02 to 2 nM of [3 H]R1881) in serum-free medium for 60 min at 37°C. The cells were washed three times with PBS to remove unbound androgen. Bound androgen was extracted from the cells using ice-cold ethanol and measured in a scintillation counter. Nonspecific binding was measured in the presence of a 400-fold excess of unlabeled R1881. Scatchard analysis was used to determine the binding affinity.

Androgen and PP2A dissociation assays in vivo. Ad-infected cells were washed three times with PBS and incubated with 2 nM [3 H]R1881 (or 100 nM ASD) in serum-free medium for 60 min at 37°C. The cells were then washed three times with PBS and incubated with 1 μ M cold R1881 or 100 μ M bicalutamide for the times indicated in the figure legends (0 to 3 h). The cells were again washed twice in PBS. The bound androgen was extracted with ice-cold ethanol, and the counts were measured in a scintillation counter. Alternatively, proteins were extracted from the cells using Triton lysis buffer followed by IP using AR441. The amount of [3 H]R1881 bound to AR was measured in a scintillation counter. Proteins isolated by IP were subjected to SDS-PAGE, and the levels of AR and PP2A were determined by immunoblotting.

Androgen/PP2A dissociation assays in vitro. AR complexes were isolated by IP from AR and ST-expressing cells treated with R1881. AR complexes immobilized on antibody-protein G beads were incubated in Triton lysis buffer at room temperature for 3 h to allow androgen and PP2A dissociation. Following centrifugation, the initial bead fraction, the postdissociation bound fraction, and the dissociated fraction were all treated with SDS-PAGE loading buffer and subjected to immunoblot analyses.

Transcription assays. Transcription was measured by use of a dual-luciferase reporter assay system (Promega). Cells were grown in RPMI PRF medium with 5% fetal bovine serum (charcoal and dextran stripped) in 12-well dishes. The cells were transfected with the indicated plasmids according to protocols appropriate for each cell type. Each well typically received a total of 245 or 345 ng of DNA, which included 145 ng of reporter plasmid and 100 or 200 ng of AR

FIG. 1. Identification of HEAT repeats in the A subunit of PP2A required for binding to AR. (A) Diagram showing the 15 HEAT repeats in the A subunit and known contact sites for ST and the C subunit. The N- and C-terminal deletions that were used to map the AR binding site are depicted. C-subunit binding to the A-subunit deletion mutant spanning HEAT repeats 6 to 15 was not determined (ND*); however, C-subunit binding to a PP2A A subunit that lacks HEAT repeats 3 to 6 was shown in a previous study (34). (B) ST binding region of the PP2A A subunit is required for PP2A A/C heterodimer binding AR. A subunits encoding HEAT repeats 3 to 15 (amino acids 84 to 589) or HEAT repeats 6 to 15 (amino acids 180 to 589) were coexpressed with AR in Cos7 cells, and the cells were incubated with 10 nM R1881 for 60 min before harvest. AR complexes were immunoprecipitated using AR441 and immunoblotted using AR21 and goat polyclonal anti-A-subunit. Deletion of HEAT repeats 3 to 5 is sufficient to disrupt ST binding to the A subunit and, therefore, ST-induced PP2A binding to AR. (C) AR binding to the PP2A A subunit is lost upon deletion of HEAT repeats 13 to 16. Flag-tagged A subunits containing the indicated HEAT repeats were coexpressed with AR as described above. IPs were carried out using AR441 or M2 anti-Flag as indicated. Antibodies used for immunoblotting were AR21, M2, and rabbit polyclonal anti-C-subunit. PP2A C-subunit binding to the A subunit is lost upon deletion of HEAT repeats 14 and 15, and AR binding to the A subunit is lost upon further deletion of HEAT repeat 13. Thus, the C-subunit and AR binding sites on the A subunit are nonidentical. (D) AR binding to the PP2A A subunit requires HEAT repeat 13. HA-tagged A subunits were coexpressed with ST in LNCaP cells, which were treated with R1881. Antibodies used were PAb430 anti-ST or 16B12 anti-HA for IP and, for immunoblotting, AR21, 16B12 anti-HA, rabbit anti-C subunit, or PAb108 anti-ST. AR binding to the PP2A A subunit is lost upon deletion of HEAT repeat 13. This deletion also results in loss of PP2A C-subunit binding to the A subunit, likely because intersubunit hydrogen binding is disrupted. Amino acids Tyr495 and Arg498 in HEAT repeat 13 form hydrogen bonds with Asn79 and Asp280 in the C subunit (5, 46). α , anti.

plasmid; the balance was empty vector pcDNA3. The luciferase reporters used in this study contained the 6-kb PSA promoter and androgen response element (ARE) I or III from PSA (7) fused to a minimal thymidine kinase promoter. After 24 h of transfection, the medium was changed, and the cells were treated with AR ligands for an additional 24 h. Cells were then washed once with PBS and lysed in passive lysis buffer (Promega). Luciferase activities in lysates were measured on a Berthold LB 953 luminometer. Firefly luciferase activities were normalized to *Renilla* luciferase activities (plotted as firefly activity/*Renilla* activity); assays were performed in triplicate, and the results are representative of at least three experiments. Error bars stand for standard deviation. Immunoblotting was used to verify that differences in transcription were not due to AR expression levels. Phosphorylation site mutants of AR were constructed with alanine substitutions (serines 81, 94, 256, 308, and 424) or with aspartate substitutions (serines 81, 94, 256, 308, 424, and 650) by a combination of PCR, restriction digestion, and ligation methods, and all were confirmed by DNA sequencing.

EMSA. Oligonucleotides representing C3(1)-ARE DNA (6) were end labeled with [³²P]dATP (Perkin-Elmer). EMSAs (20- μ l reaction mixtures) contained 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂, 10 mM R1881, 0.05% Triton X-100, 0.5 mM EDTA, 2 mM dithiothreitol, 100 pmol of single-stranded DNA, 1 μ g of poly(dI-dC), 0.05 mg/ml bovine serum albumin, 15% glycerol, and either purified AR complexes or recombinant glutathione *S*-transferase-AR(DBD-Hinge). Reactions were supershifted with an anti-AR hinge region antibody (1 μ g). Reaction mixtures were preincubated at room temperature for 5 to 10 min, and AR-ARE binding was initiated by the addition of the labeled probes (~40,000 cpm/reaction). The reaction mixture was incubated at room temperature for 15 to 20 min and analyzed by nondenaturing PAGE (4% gel). The gel was run in 0.25 $\times$ Tris-borate-EDTA buffer and run at 100 V at 4°C. The gels were vacuum dried on 3MM paper and analyzed by using a phosphorimager. There is no supershift of the AR complexes in the assays using the antibodies against the PP2A A subunit or C subunit, probably because none of these antibodies recognizes native protein by IP (our unpublished observations).

Xenograph analysis. LNCaP cell lines stably expressing SV40 ST, ST-myc, or vector alone were prepared by hygromycin selection. LNCaP cell lines (5 $\times$ 10⁶ cells) were mixed with 100 μ l of matrigel and injected subcutaneously into the flanks of 6- to 8-week-old intact male nude mice (NCI athymic nude mice). Tumors were grown for 11 to 16 weeks, and tumor size was monitored by caliper measurements. Some mice were surgically castrated 4 days prior to tumor harvest. Tumors were cut into small pieces, ground in liquid nitrogen using a mortar and pestle, and resuspended in 5 volumes of homogenization buffer (250 mM sucrose, 50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 5 μ g/ml each of aprotinin, leupeptin, and pepstatin A). The mixture was homogenized on ice using a hand-held polytron (five 2-s pulses with 10-s pauses between bursts). Homogenates were separated into supernatant and pellet fractions by centrifugation (18,000 $\times$ g for 15 min at 4°C). The supernatant was supplemented with 0.5% Triton X-100 and then subjected to IP using anti-AR antibody G122-134 and AR441 (1:1). The AR complexes were separated by SDS-PAGE and analyzed by immunoblotting.

RESULTS

PP2A heterodimer binding to AR is mediated by the structural A subunit, a protein that is composed of 15 HEAT repeats arranged in tandem (17). To determine which HEAT repeats within the PP2A A subunit are responsible for binding to AR, we generated a series of deletion mutants that omit HEAT repeats from the N terminus, the C terminus, or both (Fig. 1A). The HEAT repeat deletion mutants of the PP2A A subunit were coexpressed with AR in Cos7 cells, which express ST. Following androgen treatment of the cells, the AR complexes were isolated by IP and examined by immunoblotting for the presence of PP2A. In this co-IP assay, transfected PP2A A subunit lacking HEAT repeats 1 and 2 (retains amino acids 84 to 589) bound to AR, but further removal of HEAT repeats 3 to 5 (retains amino acids 180 to 589) generated a form that was inactive for binding to AR (Fig. 1B). Because ST binds HEAT repeats 3 to 6 (34), this result corroborates our previous finding that ST contact with the A subunit is necessary for PP2A transfer onto the androgen-bound form of AR (48).

PP2A binding to AR requires A-subunit HEAT repeat 13. We used the PP2A A subunit lacking HEAT repeats 1 and 2 to generate a series of C-terminal deletion mutants for further analysis. Deletion of HEAT repeat 15 (retains amino acids 84 to 547) from this construct had no apparent effect on ST-induced PP2A binding to AR (Fig. 1C). Further removal of HEAT repeat 14 (retains amino acids 84 to 516) from the A subunit resulted in the loss of C-subunit binding without affecting A-subunit binding to AR (Fig. 1C). Removal of HEAT repeats 13 to 15 (retains amino acids 84 to 478) resulted in a complete loss of PP2A A-subunit binding to AR. Our data suggest that the region of the A subunit that binds AR includes HEAT repeat 13, which is distinguishable from the PP2A C-subunit binding site that requires HEAT repeat 14.

We constructed a mutant form of PP2A A subunit that lacks only HEAT repeat 13. The HEAT repeat 13 deletion mutant of the PP2A A subunit interacted with ST, but it did not undergo transfer onto androgen-bound AR (Fig. 1D). Thus, HEAT repeat 13 is critical for AR to bind the PP2A A subunit. Deletion of HEAT repeat 13 also disrupted C-subunit binding to the A subunit (Fig. 1D). This result is not surprising, given the biochemical and crystallographic evidence that the C subunit directly contacts HEAT repeats 11 to 15 in the A subunit (5, 35).

PP2A transfer reaction is highly specific for SV40 ST. We investigated whether a homologous ST from human polyomavirus can substitute for SV40 ST in mediating PP2A transfer to AR. JCV causes multifocal leukoencephalopathy in brain, but it is also found in other tissues including the prostate (49). Under conditions where SV40 ST induced PP2A transfer to AR, transient JCV ST expression did not result in PP2A binding to AR (Fig. 2A). Although JCV ST is 65% identical to SV40 ST and we determined that JCV ST binds the PP2A A subunit (Fig. 2B), it fails to induce the A-subunit conformation that is necessary for PP2A transfer to AR. We obtained the same result whether antibodies were used to immunoprecipitate AR or HA-tagged A subunit (Fig. 2C).

The ability of SV40 ST to bind the PP2A A subunit and modulate targeting of the A/C heterodimer to substrates destined for dephosphorylation is a function usually mediated by PP2A B subunits. To explore whether known B subunits can promote PP2A targeting to AR in a manner analogous to the reaction mediated by ST, we tested five major B subunits in the co-IP assay. Overexpression of any one of these B-subunits (α , β , γ , δ , or ϵ), however, did not promote PP2A transfer to AR (Fig. 2D). The B subunit from the B' family (B' α) also did not promote PP2A loading onto AR (Fig. 2E).

PP2A interaction with the AR LBD. AR lacking the AF-1 domain is functional for PP2A binding, but further deletion of the DBD results in a loss of PP2A binding (48). We interpreted this as evidence that PP2A binding to AR may be dependent on the DBD, either because the DBD provides part of the PP2A binding site or because the DBD stabilizes LBD structure. To distinguish between these possibilities, we deleted the DBD from AR (Δ DBD) and tested it for PP2A binding in the co-IP assay. In the presence of ST and androgen, PP2A was transferred to full-length AR and to the Δ DBD form of AR but not to the Δ LBD form of AR (Fig. 3A). This result together with previous mapping data indicates that the LBD is necessary for PP2A binding, and it rules out an essential func-

FIG. 2. PP2A transfer onto AR is specific for SV40 ST. (A) The closely related ST from JCV does not mediate PP2A transfer onto AR. JCV ST and SV40 ST were expressed in LNCaP cells, which were treated with R1881. Antibodies used were AR441 for IP and, for immunoblotting, PAb108 anti-ST (for both SV40 and JCV ST), AR21, goat anti-A subunit, and rabbit anti-C subunit. (B) JCV ST interacts with the PP2A A subunit. JCV ST was expressed in LNCaP cells in the presence or absence of the HA-tagged A subunit. Cells were treated with R1881. Antibodies used were 16B12 anti-HA for IP and, for immunoblotting, 16B12 anti-HA and PAb108 anti-ST. (C) HA-tagged A subunit is transferred onto AR in response to ST and androgen. HA-tagged A subunit was coexpressed with JCV ST or SV40 ST in LNCaP cells. Cells were treated with R1881. Antibodies used were AR441 and 16B12 α -HA for IP and, for immunoblotting, PAb108 anti-ST, 16B12 anti-HA, and AR21. (D) Overexpressed PP2A B subunits do not mediate PP2A transfer onto AR. HA-tagged B-subunits (α , β , γ_1 , δ , and ϵ) were coexpressed with AR in 293 cells. Cells were treated with R1881. Antibodies used were 16B12 α -HA for IP and, for immunoblotting, 16B12 anti-HA, goat anti-A subunit, and AR21. These B subunits bind to the A subunit of PP2A but do not induce the conformation required for PP2A transfer onto AR. (E) PP2A B' α subunit does not mediate PP2A transfer onto AR. Flag-tagged B' α subunit was expressed in LNCaP cells. Cells were treated with R1881. Antibodies used were AR441 for IP and, for immunoblotting, M2 anti-Flag, rabbit anti-C subunit, and AR21. α , anti.

tion for the DBD in this interaction. As the LBD alone is not sufficient for PP2A binding, we infer that the agonist conformation that is recognized by PP2A requires a conformation that is stabilized by interdomain interactions that can be provided by either the N-terminal AF-1 region or the DBD.

To determine whether PP2A affects androgen binding or dissociation from the AR LBD, we performed androgen-binding assays in live cells using radiolabeled synthetic androgen ($[^3\text{H}]\text{R1881}$). The affinity of AR for androgen ($K_d = 0.13$ nM) following AR expression in PC-3 cells (Fig. 3D, -ST) was comparable to values reported by other laboratories (26). Coexpression of ST to promote PP2A transfer onto AR (Fig. 3D, +ST) had virtually no effect on androgen affinity ($K_d = 0.10$ nM). The apparent increase in total androgen binding (x intercept) in the presence of ST is probably due to a slightly higher level of AR expression in these samples (Fig. 3C). PP2A binding caused a slight increase in the rate of androgen dissociation from AR, decreasing the dissociation rate from a $t_{1/2}$ of 62 min to a $t_{1/2}$ of 50 min (Fig. 3E). While PP2A binding to AR requires the LBD and ST-mediated transfer is strictly dependent on the androgen-bound form of AR, the presence of PP2A in the AR complex has a relatively small effect on androgen dissociation from AR.

Effect of PP2A on AR binding to the ARE. ST expression inhibits AR-dependent transcription from the PSA promoter (48). To determine whether the inhibitory effect of ST is due to a reduction in AR binding to DNA, we analyzed AR complexes isolated by immunoaffinity (Fig. 4A and B) in an EMSA. AR binding to DNA was analyzed with the well-characterized C3(1) ARE (6) and quantified on a phosphorimager. Native AR complexes isolated from cells without or with ST coexpression, and consequently with or without bound PP2A, shifted the C3(1) ARE, and both types of complexes were supershifted by an antibody raised against the hinge region of AR (Fig. 4B and C). However, the amount of C3(1) ARE shifted by the AR-PP2A complex was about 68% of the amount shifted by the AR complex that lacked the PP2A heterodimer. The result shown is representative, as the average amount of binding in the presence of PP2A in four experiments was $67.5\% \pm 10.4\%$. The fact that the presence of PP2A does not induce a gel shift resolved in this system suggests that either the size of the heterodimer (A plus C subunit, 100 kDa) is inconsequential relative to the size of the native AR complex or that PP2A has dissociated from the AR-DNA complex. The small reduction in DNA binding could be due to AR dephosphorylation or a structural effect of PP2A binding.

Depending on the site examined, serine-to-alanine mutations of individual phosphorylation sites in AR have little or no effect on transcription activity, making it unclear how phosphorylation contributes to the transactivation function of AR (16, 31, 45). Reasoning that multisite phosphorylation of the AF-1 region could be important for AR transcriptional activity and that multisite dephosphorylation provides the basis of PP2A inhibition of AR, we tested the effect of mutating all five sites that are dephosphorylated by PP2A following ST-dependent transfer (48). For this analysis we used luciferase reporters containing the complete PSA promoter and relatively weaker promoters based on AREs I and III from the PSA promoter. AR that contained alanine mutations at five sites (Ser81Ala, Ser94Ala, Ser256Ala, Ser308Ala, and Ser424Ala)

FIG. 3. PP2A binding and dephosphorylation is dependent on the LBD of AR. (A) The LBD of AR is required for PP2A binding. Full-length AR and mutants lacking DBD (residues 559 to 616) or LBD (residue 710 to end) of AR were expressed in Cos7 cells. Cells were treated with R1881. Antibodies used were AR441 for IP and, for immunoblotting, rabbit anti-C subunit and AR21. In this reaction, ST expressed by the SV40-transformed Cos7 cells binds to endogenous A subunit and mediates transfer of the PP2A A/C heterodimer. Only the blotting for the PP2A C subunit is shown. (B) The LBD of AR is required for dephosphorylation of the AF-1 phospho-site Ser81. 293 cells were transfected with AR or the Δ LBD derivative of AR and, where indicated, ST. Cells were treated with R1881. Antibodies used were G122-434 anti-AR and AR441 (1:1) for AR IP and AR21 and rabbit anti-pSer81 for immunoblotting. ST induces PP2A binding to AR and dephosphorylation of Ser81 (lane 2) in the AF-1 region of AR (48). Upon deletion of the AR LBD, PP2A fails to bind and dephosphorylate AR (lane 4). (C) Expression levels of AR and ST used for androgen binding assays. Ad expressing AR was used to infect PC-3 cells in the presence or absence of Ad expressing SV40 ST. Antibodies used were anti-AR hinge region, PAb108 anti-ST and anti-tubulin for immunoblotting. (D) Scatchard analysis showing that PP2A binding does not affect AR affinity to androgen. PC-3 cells were infected by Ad-HisAR in the absence or presence of Ad-ST; the latter was used to promote PP2A transfer onto AR in the presence of androgen. Cells were incubated with various concentrations of [3 H]R1881 (0.02 to 0.2 nM) for 60 min and then washed with PBS. Androgens were extracted

(Fig. 4D to F, 5Aa) or aspartate mutations at six sites (Ser81Asp, Ser94Asp, Ser256Asp, Ser308Asp, Ser424Asp, and Ser650Asp) (Fig. 4D to F, 6Asp) displayed basal and androgen-induced activity levels that were comparable to WT AR. These data suggest that the reduction in AR activity that is correlated with PP2A binding cannot be explained simply by PP2A-mediated dephosphorylation of known AF-1 sites in AR.

Androgen dissociation correlates with PP2A release from AR. Since androgen-free AR does not bind PP2A, we hypothesized that structural changes that occurred in the LBD after androgen dissociation might provide the basis for PP2A release from AR. To test this hypothesis, we designed a cell-based assay that measures the rates of both androgen and PP2A dissociation from AR. Cells expressing AR and ST were initially incubated with [3 H]R1881 to allow androgen and PP2A binding. The cells were then treated with excess anti-androgen bicalutamide for up to 180 min. A large excess of bicalutamide was added to the cells during the chase period since the bicalutamide-bound form of AR does not bind to PP2A (48). This rendered the reaction unidirectional and allowed us to measure the dissociation rate of both androgen and PP2A from AR in vivo. AR complexes isolated at each time point by IP were analyzed by immunoblotting for AR and PP2A content (Fig. 5A) and by scintillation counting for bound radiolabeled androgen (Fig. 5B). The data for androgen dissociation and PP2A dissociation were each fit with a single exponential decay curve, indicating that both dissociation reactions obey first-order dissociation kinetics (Fig. 5B and C). There is a statistically significant correlation ($R^2 = 0.986$) between the amounts of PP2A and androgen that are bound to AR during the time course of dissociation (Fig. 5D). Our data suggest that androgen dissociation is correlated with PP2A dissociation from AR, but we considered the formal possibility that PP2A remained bound to the androgen-free AR and that bicalutamide binding induced a conformation in the LBD that stimulated PP2A release. We addressed this possibility by showing that PP2A dissociates from AR in the absence of bicalutamide in vitro (Fig. 5E) to an extent that is comparable to that measured in the presence of bicalutamide in vivo (Fig. 5A).

If PP2A dissociation from AR is coupled to androgen dissociation, then increasing the rate of androgen dissociation should result in an increase in the rate of PP2A dissociation. We tested this prediction using ASD, an androgen that binds AR with an affinity that is >200-fold lower than R1881 (3). Cells expressing AR and ST were incubated with ASD to allow PP2A binding. The cells were then treated with excess anti-

and measured in a scintillation counter. Nonspecific binding was measured in the presence of a 400-fold excess of unlabeled R1881 and subtracted. Based on immunoblotting, the difference in total androgen binding likely reflects differences in AR expression levels. The results are representative of four experiments. (E) PP2A binding to AR has a small effect on androgen dissociation. PC-3 cells were infected by Ad-HisAR in the absence or presence of Ad-ST; the latter was used to promote PP2A transfer onto AR in the presence of androgen. Cells were incubated with 2 nM [3 H]R1881 for 60 min, washed with PBS, and then chased with 1 μ M cold R1881 for the indicated time periods. Androgens were extracted and measured in a scintillation counter. The results are representative of three experiments. AU, arbitrary units.

FIG. 4. AR Complexes analyzed by EMSA and analysis of multisite phosphorylation mutants of AR. (A) AR complexes used for EMSA. PC-3 cells were infected with Ad-HisAR in the absence or presence of Ad-ST and treated with 10 nM R1881. AR complexes were isolated by IP using AR441, eluted with specific peptide in the presence of androgen, and analyzed by silver staining or immunoblotting. Antibodies used for immunoblotting were anti-AR hinge region, goat anti-A subunit, and rabbit anti-C subunit. (B) EMSA using C3(1)-ARE oligonucleotides and purified AR complexes. The arrows indicate the positions of protein-DNA complexes, which are supershifted in the presence of anti-AR antibody. When the reaction is performed using the AR-PP2A complexes, binding to the ARE DNA is reduced to 68% of that observed with AR complexes. Because a PP2A-dependent gel shift of the AR-ARE complex is not resolved under these conditions, the total mass of the PP2A heterodimer (~100 kDa) is insufficient to give a supershift, or PP2A has dissociated from the AR-DNA complex. (C) Specificity of the antibody used for EMSA. Affinity purified anti-hinge region Ab (epitope: amino acids 656 to 669) induces supershift of a fragment encoding the AR DBD-hinge. (D to F) Transcription assay of WT and multisite mutants of AR measured in PC-3 cells using the indicated promoters. Error bars represent standard deviations. Immunoblotting was used to confirm that similar levels of WT and mutant AR proteins were expressed (not shown). 5Ala, Ser81Ala, Ser94Ala, Ser256Ala, Ser308Ala, and Ser424Ala; 6Asp, Ser81Asp, Ser94Asp, Ser256Asp, Ser308Asp, Ser424Asp, and Ser650Asp; LUC, luciferase.

androgen bicalutamide for up to 90 min (Fig. 6). We found that the $t_{1/2}$ for dissociation of the AR-PP2A complex assembled in the presence of ASD is 18.5 min. This is significantly shorter than the $t_{1/2}$ for dissociation of the AR-PP2A complex assembled in the presence of R1881, which was determined to be 70 min (Fig. 5). The actual dissociation rate of ASD has not been reported; however, the low affinity for AR indicates that ASD must have a faster off rate than R1881. Our finding that PP2A dissociation from AR is faster when the AR-PP2A com-

plex is assembled using an androgen with a faster dissociation rate supports our model that androgen dissociation is linked to PP2A dissociation.

At least two biochemically distinct conformations of AR are competent for transcription. Certain LBD mutations are known to broaden the range of ligands recognized by AR such that androgen antagonists can function as agonists. For example, the most commonly occurring AR LBD mutation in prostate cancer is T877A, which renders AR responsive to the

FIG. 5. PP2A release from AR is correlated with R1881 dissociation. (A) AR-PP2A dissociation analyzed in vivo. AR-PP2A complexes were assembled in PC-3 in the presence of ST by the addition of a 2 nM concentration of radioactive [³H]R1881 (R1881 pulse). The cells were washed with PBS and then treated with 100 μM bicalutamide (Casodex chase) for the indicated time periods. AR-PP2A complexes were isolated by IP using AR441 and eluted in SDS-PAGE sample buffer, and [³H]R1881 bound to AR was measured by scintillation counting. AR and PP2A were quantified by immunoblotting using anti-AR hinge region and rabbit anti-C subunit antibodies. (B) AR-androgen dissociation rate analyzed in vivo. Androgen dissociation from AR follows first-order kinetics ($R^2 = 0.978$). (C) Plot of the AR-PP2A dissociation data shown in panel A. The data were normalized to the amount of AR recovered in each IP and show that the loss of PP2A from AR follows first-order kinetics ($R^2 = 0.924$). (D) Plot showing a correlation between the amount of PP2A and androgen bound to AR during the dissociation reaction ($R^2 = 0.986$). (E) Androgen dissociation from AR (in the absence of antagonist) results in PP2A dissociation in vitro. The AR441-protein G beads containing

FIG. 6. PP2A dissociates rapidly from AR complexes assembled in the presence of ASD. (A) AR-PP2A complexes were assembled in PC-3 cells in the presence of ST by the addition of 100 nM ASD, which has a much weaker affinity (>200-fold) for AR (3). Cells were washed with PBS and then treated with 100 μM bicalutamide (Casodex chase) for indicated time periods. Antibodies used were AR441 for IP and anti-AR hinge region and rabbit anti-C subunit for immunoblotting. (B) Plot of AR-PP2A dissociation using the data shown in panel A. The data were normalized to the amount of AR recovered in each IP and show that the loss of PP2A from AR follows first-order kinetics ($R^2 = 0.993$). AU, arbitrary units.

androgen antagonists such as HO-Flut (13, 42). We set out to determine if androgen antagonists can, in the context of the T877A mutation, promote formation of an androgen-like agonist conformation in AR by using PP2A binding as the read-out. We first confirmed that the T877A mutation in AR enhances transactivation measured from PSA and mouse mammary tumor virus (MMTV) promoters in response to androgen antagonists Flut, HO-Flut, and to a lesser extent, bicalutamide (Fig. 7A and B) (13, 38, 42). These ligands did not appear to selectively stabilize T877A AR levels (Fig. 7C and data not shown). This argues that the enhancement of AR-dependent transcription measured with the T877A mutant can be ascribed to ligand-dependent activation of transcription and not simply AR protein stabilization. We examined whether androgen antagonists and androgen-related ligands promote an androgen-bound conformation in T877A AR by transient transfection of WT and mutant AR proteins in Cos7 cells. Analysis of the IP products revealed that the androgen antagonists that result in robust transactivation of T877A AR do so without promoting an AR conformation that binds PP2A (Fig. 7C). The same was true for the adrenal androgen DHEA and for estradiol. High concentrations of the adrenal androgen ASD, like DHT, and synthetic androgen R1881 promote an

purified AR-PP2A complexes (the initial bead fraction) were incubated with a large volume of Triton lysis buffer at room temperature for 180 min and then separated into the remaining bead fraction (bound) and the supernatant fraction (released). AR and PP2A in the initial bead fraction was compared with different gel loadings (lanes 1 to 5), the remaining bead fraction (lane 6), and the released supernatant fraction (lane 7). AU, arbitrary units.

FIG. 7. At least two distinct ligand-dependent conformations of AR can mediate transcription. (A) AR-dependent transcription from the PSA promoter stimulated by androgen agonists and antagonists. PC-3 cells transfected with the PSA-luciferase (PSA-Luc) reporter plasmid and either WT AR (open bars) or T877A AR (gray bars), treated with the indicated ligands, and normalized to CMV-*Renilla*. (B) AR-dependent transcription from the MMTV promoter stimulated by androgen agonists and antagonists. PC-3

AR conformation that stimulates transactivation and supports PP2A binding (Fig. 7A, B, and C).

We examined the capacity of these ligands to promote expression of the endogenous PSA gene in LNCaP cells, which express a mutant form of AR (T877A) that has a broader ligand responsiveness. LNCaP cells were treated with ligands for 24 h and harvested for immunoblot analysis of PSA. Similar to the results obtained with nonintegrated luciferase reporters, ligands that promoted the agonist conformation (DHT and ASD) were highly effective at activating endogenous PSA expression (Fig. 7D). Certain ligands (HO-Flut and estradiol) that failed to promote the androgen-bound conformation were effective at turning on PSA expression. An interesting difference between reporter and endogenous PSA gene activation was noted using the adrenal androgen DHEA. AR transactivation (for both wild type [WT] and T877A) measured using reporters in the presence of DHEA equaled or exceeded the levels of AR transactivation obtained with DHT. In contrast, PSA gene expression measured in the presence of DHEA was less than the levels of PSA gene expression in the presence of DHT, ASD, HO-Flut, and estradiol (Fig. 7D). Our results indicate that a non-androgen-bound conformation of AR can promote efficient transcription from both reporter and endogenous genes. The increase in AR activity observed with both reporter and endogenous genes depends on the particular ligand and whether the AR LBD is WT or mutant.

ST-dependent PP2A transfer to AR in a xenograft model of prostate cancer. We prepared stable lines of LNCaP cells expressing ST for xenograft studies that would enable us to test whether androgen status influences PP2A loading onto AR in vivo. ST-myc-expressing cells were generated as a control line, since a minimal C-terminal myc tag inhibits the PP2A transfer function of ST (48). We used IP analysis to verify that PP2A transfer onto AR in the stable lines grown in culture requires androgen (R1881) and functional ST (Fig. 8A). The cell lines were next grown as subcutaneous tumors in nude mice, and 4 days prior to tumor harvest, a subset of mice bearing LNCaP-ST tumors was castrated to deplete testicular androgens. This would allow us to test whether a PP2A transfer reaction could occur in the presence of ST in the context of an animal model and whether AR can adopt an androgen-bound conformation in an animal following castration. AR complexes were isolated from the tumor extracts by IP and then probed for the presence of AR-PP2A complexes. As a functional read-

cells transfected with the MMTV-Luc reporter plasmid and either WT AR (open bars) or T877A AR (gray bars) and assayed in the absence and presence of the indicated ligands as above. (C) PP2A binds to the DHT-bound and ASD-bound forms of WT and T877A AR. Cos7 cells were transfected with AR and treated with the indicated androgens or androgen antagonists. AR affinity purification was carried out using AR441. AR complexes were analyzed by immunoblotting using anti-AR hinge region and rabbit anti-C subunit. (D) PSA induction in LNCaP by different ligands. LNCaP was used because it expresses the T877A mutant form of AR and endogenous PSA. LNCaP cells grown in RPMI PRF medium plus 5% charcoal- and dextran-stripped fetal bovine serum were incubated with indicated ligands for 24 h. Cells were washed with PBS and extracted with SDS-PAGE sample buffer. Extracts were analyzed by immunoblotting using anti-AR hinge region, anti-tubulin, and anti-PSA antibodies.

FIG. 8. ST mediates PP2A transfer onto AR in LNCaPs grown as xenografts in mice. (A) Characterization of LNCaP cells stably expressing ST and ST-myc. Cells were incubated with R1881 for 60 min. IP was carried out using AR441, and immunoblotting was performed with anti-AR hinge region, goat anti-A subunit, and PAb108 anti-ST. (B) Analysis of LNCaP cells stably expressing ST and ST-myc after growth as xenografts in nude mice. Tumor growth, castration, tumor homogenization, and protein extraction were carried out as described in Materials and Methods. AR complexes were purified using G122-434 anti-AR and AR441 (1:1). Antibodies used for immunoblotting were PAb108 anti-ST, anti-AR hinge region, rabbit anti-C subunit, and a panel of phospho-site-specific antibodies (48). The effect of castrate levels of androgen on ST-dependent PP2A transfer and AR dephosphorylation state was examined in mice subjected to castration (Castr) prior to tumor harvest. α , anti.

out of PP2A binding to AR, we also examined the phosphorylation state of phospho-sites in the AR AF-1 region that are dephosphorylated by PP2A in cells grown in tissue culture. In the LNCaP-ST xenograft harvested from the intact mouse, the C subunit of PP2A was present in the AR complex, indicating that ST mediates PP2A transfer to AR in this model system (Fig. 8B, lane 2). ST-myc failed to mediate PP2A transfer onto AR (Fig. 8B, lane 1), which is consistent with results obtained in cultured cells (48). The presence of PP2A in the AR complex was correlated with a reduction in AF-1 phosphorylation. Unexpectedly, the level of PP2A bound to AR from the LNCaP-ST tumor from the castrated mouse was comparable to the level observed in the tumor from the intact mouse (Fig. 8B, lane 3). Thus, under castration conditions, while castrate levels of androgen in the nude mouse are insufficient to maintain growth of a number of androgen-dependent cell lines and xenografts, AR in tumor xenografts is still capable of forming a conformation that can bind stably to PP2A.

This shows that in LNCaP cells propagated as a xenograft, AR acquires a conformation that, by the biochemical criterion of PP2A binding, is like the conformation induced by DHT, R1881, and ASD. This suggests that castrate levels of androgen (including testicular androgen DHT and testosterone and adrenal androgen ASD) are sufficient for driving the androgen-bound conformation of AR. It is also possible that the milieu provided by the xenograft allows activation of signaling pathways that promote the androgen-bound conformation of AR.

AR phosphorylation sites are kinase accessible prior to androgen binding. There is a well-established correlation between androgen binding, conformational changes, and enhanced phosphorylation of at least five serines in AR (16, 24). A logical interpretation of these data is that ligand-induced conformational changes in AR are important for kinases to access phosphorylation sites that are otherwise inaccessible. Our finding that the conformation of AR has a profound influence on its interaction with the phosphatase PP2A (in cells expressing ST), however, led us to consider an alternative model to explain how the phosphorylation state of AR is regulated. We hypothesized that the more highly phosphorylated state of AR observed in response to androgen binding might have resulted from a reduced interaction between AR and phosphatases. According to this hypothesis, androgen-free AR would be subject to a constant cycle of phosphorylation and dephosphorylation but would be maintained in a low phosphorylation state. In the presence of androgen, however, AR phosphorylation would predominate because the conformation induced by androgen binding is less compatible with efficient phosphatase binding and dephosphorylation.

We tested whether known phospho-sites are accessible to kinases by expressing AR in 293 cells and treating the cells with androgen and/or the phosphatase inhibitor OA. The AR was then isolated by IP and analyzed using phospho-site-specific antibodies. As shown previously, androgen treatment increased the phosphorylation level of Ser81, Ser256, Ser308, and Ser424 (Fig. 9A, +R1881) (16, 48). The phosphorylation levels of Ser16 and Ser94 displayed little change with androgen treatment, consistent with apparent constitutive phosphorylation of these sites (48). Remarkably, the level of phosphorylation of all AF-1 sites in AR showed a large increase in the presence of a concentration of OA (400 nM) that inhibits phosphatases PP2A and PP5 (Fig. 9A, lane 3). Cells treated with OA and androgen (Fig. 9A, lane 4) showed little additional increase in the level of AR phosphorylation. From these data, we conclude that AF-1 phosphorylation sites in AR are kinase accessible prior to androgen binding. PP2A binding to AR does not preclude kinase access to the sites since AR-PP2A complexes are highly phosphorylated in the presence of OA (Fig. 9C).

The AR LBD regulates phosphatase access to AF-1 sites. We addressed whether the LBD represses phosphatase access by deleting the LBD and examining the effect on AR phosphorylation. Ser16 and Ser94 in the full-length AR were constitutively phosphorylated sites that were not induced by the addition of androgen; deletion of the LBD had no effect on these two sites (Fig. 9B). Two sites that show a robust induction of phosphorylation by androgen, Ser81 and Ser308, were phosphorylated in the Δ LBD form of AR that was comparable to the level of phosphorylation in full-length AR induced by an-

FIG. 9. The phosphorylation state of the AF-1 is governed by conformational changes in AR that regulate phosphatase action. (A) Phosphorylation sites in AR are kinase accessible in androgen-free AR. 293 cells transfected with AR were treated with R1881 (10 nM for 1 h), OA (400 nM for 3 h), or the two combined (OA for 2 h and then OA plus R1881 for 1 h). AR complexes were isolated using G122-434 anti-AR and AR441 (1:1). Immunoblotting was performed using anti-AR hinge region and a panel of phosphoserine antibodies. Treating cells with a concentration of OA that is sufficient to inhibit PP2A and PP5 results in a high level of AF-1 phosphorylation at multiple sites. (B) OA inhibits the catalytic activity of PP2A but it does not affect PP2A binding to AR. 293 cells cotransfected with AR and ST were subjected to drug treatments and IP as described for panel A. Antibodies used for immunoblotting were anti-AR hinge region, goat anti-A subunit, rabbit anti-C subunit, and a panel of phosphoserine antibodies. The presence of OA does not affect the transfer reaction, and the concentration of OA used is capable of inhibiting PP2A in the AR complexes. (C) Deletion of LBD results in phosphorylation at several phospho-sites in the AF-1 region of AR. 293 cells were transfected with WT AR or mutant AR lacking the LBD (deletion of residues 710 to 919). Cells were treated with 10 nM R1881 for 2 h, where indicated. AR complexes were isolated using G122-434 anti-AR and AR441 (1:1). Immunoblotting was performed using anti-AR hinge region and a panel of phospho-site antibodies.

drogen (Fig. 9C). Also, Ser424 phosphorylation in the Δ LBD form of AR was phosphorylated to the same extent as in full-length AR. In contrast to these sites, the level of phosphorylation of Ser256 was clearly not induced when the LBD was deleted. We interpret these data as evidence that the LBD of AR is critical for efficient phosphatase interaction with AR because its removal results in constitutive phosphorylation of at least two sites that are subject to dephosphorylation in

androgen-free AR. Thus, androgen binding either reduces phosphatase targeting to AR or induces an AR conformation that protects Ser81 and Ser308 from dephosphorylation. Phosphorylation of Ser256 is strongly dependent on the LBD for reasons related either to phospho-site conformation or kinase targeting to AR or simply because Ser256 in Δ LBD AR is dephosphorylated more efficiently than it is phosphorylated.

DISCUSSION

Androgen binding regulates multiple aspects of AR function, including subcellular localization, interactions with transcriptional regulators, and protein degradation (10, 28, 36, 44). Ligand binding regulates these activities by altering AR conformation, chaperone composition, and protein interactions (20). In the current study we have analyzed several aspects of AR structure and activity that are linked to androgen-dependent conformational changes. The entrée for these studies was our recent finding that the phosphatase PP2A recognizes the agonist conformation of AR (48), an interaction that we have characterized in the present study.

Mechanism of PP2A binding to AR. The PP2A core enzyme is a heterodimer that contains a catalytic C subunit and a structural A subunit. In previous work we showed that the A subunit of PP2A contacts AR, and we presented data suggesting that ST-induced conformational changes in the A subunit are critical for PP2A A/C heterodimer to bind the androgen-bound form of AR (48). ST binds directly to the A subunit (34), and we found that ST binding to the A subunit in vitro alters the protease sensitivity of sites both within and outside of the ST interaction site (48). Work from other laboratories had mapped the ST binding site to HEAT repeats 3 to 6 in the N-terminal region of the A subunit (34). We found that deletion of HEAT repeats 3 to 5 abrogated ST-induced PP2A binding to AR (Fig. 1), suggesting that HEAT repeats 3 to 5 (or the interface between HEAT repeats 3 and 5) are critical elements of the ST binding site (Fig. 10A). Our deletion analysis also revealed that removal of HEAT repeats 13 to 15 in the C-terminal region of the A subunit eliminates PP2A binding to AR. Moreover, we determined that deletion of HEAT repeat 13 is sufficient to disrupt PP2A A-subunit binding to AR. Together, these results indicate that ST binding to N-terminal HEAT repeats elicits a conformational change in the C-terminal HEAT repeats that creates binding site for AR.

The PP2A C subunit also binds to C-terminal HEAT repeats in the A subunit; its binding site overlaps, but is nonidentical to, the binding site for AR. This conclusion is based on two observations. First, we can isolate an AR complex that contains both the A subunit and the C subunit of PP2A, and the levels of the three polypeptides are approximately stoichiometric (48). Second, deletion of HEAT repeats 14 to 15 from the A subunit causes the loss of C-subunit binding without affecting AR binding. Removing HEAT repeat 13 from the A subunit, however, resulted in loss of both AR and C-subunit binding. Recent crystal structures of PP2A show that several residues within HEAT repeat 13 of the A subunit are important for the interface with the C subunit (5, 46). The PP2A A subunit binds stably to a site that includes the LBD of AR, thereby functioning as a targeting subunit for the PP2A C subunit. The ability of the PP2A C subunit to dephosphorylate five phospho-sites

FIG. 10. Protein conformation regulates phosphatase action on AR. (A) Model of ST-induced conformation change in the PP2A A subunit based on the structure solved by Barford and colleagues (Protein Data Bank entry 1B3U). Superimposition of noncrystallographic related copies of the PP2A A subunit emphasizes the regions of conformational flexibility, as noted previously (17). The A chain (blue and magenta; opaque) and the B chain (pink and cyan; transparent) were aligned using PyMOL (12). While the A and B chains show a high degree of alignment in HEAT repeats 4 to 12, the displacement of helices in HEAT repeats 1 to 3 and 13 to 15 in these structures suggests conformational flexibility in the N- and C-terminal regions, respectively. ST binding at the ST interaction site is proposed to induce a conformational change that is propagated N to C through the A subunit; this is manifest as an AR interaction site. In turn, AR binding is proposed to induce a conformational change that is propagated C to N that promotes release of ST. The biochemical evidence for this model is that ST induces PP2A binding to AR, but ST is fully dissociated from the PP2A-AR complex (48). (B) Two models summarizing how ligand binding could regulate the phosphorylation state of AR in cells in the absence of ST. In the kinase access model, ligand-regulated phosphorylation sites are kinase inaccessible due to AR conformation or masking by chaperones. Androgen binding induces structural changes in AR that reveal the ligand-regulated phosphorylation sites. In the phosphatase access model, AR phosphorylation is regulated by the actions of both kinases and phosphatases. Under ligand-free conditions, phosphatase action predominates, resulting in a lower level of AR phosphorylation. Ligand binding reduces phosphatase action on AR and increases AR phosphorylation. Phosphatase action on AR could be reduced because the phosphatase targeting mechanism is lost upon ligand binding. For example, if phosphatase targeting depends on AR-associated Hsp90, ligand-induced Hsp90 dissociation would reduce the AR-phosphatase interaction. Alternatively, ligand binding

in the AF-1 region (Ser81, Ser94, Ser256, Ser308, and Ser424) implies that the PP2A heterodimer has conformational flexibility when bound to AR.

Crystallographic studies of PP2A (5, 17, 46) provide a context for considering how ST contact with the A subunit might facilitate PP2A binding to the AR. Each of the 15 HEAT repeats in the PP2A A subunit consists of anti-parallel A and B helices that are connected by a short turn. The HEAT repeats are stacked such that the A helix in HEAT repeat 2 contacts the A helix in HEAT repeats 1 and 3, and so forth. Likewise, the B helix in the HEAT repeat 2 contacts the B helix in HEAT repeats 1 and 3. The overall structure is an elongated, left-handed superhelix with A helices on the convex surface and B helices on the concave surface with dimensions of ~ 45 Å by 65 Å (17). The approximately parallel arrangement of A helices with A helices (and B helices with B helices) is not, however, maintained throughout the entire superhelix. Discontinuities in helix stacking are found between HEAT repeats 3 and 4 and between HEAT repeats 12 and 13. Because of these discontinuities, the PP2A A subunit can be divided into three regions consisting of HEAT repeats 1 to 3, 4 to 12, and 13 to 15, each of which is believed to form a stable structural unit (17). As shown in Fig. 10A, superimposition of two A-subunit molecules from the crystallographically asymmetrical unit reveals a striking degree of alignment of HEAT repeats 5 to 12, but there is apparent conformational flexibility of the N-terminal and C-terminal HEAT repeats. The apparent flexibility of HEAT repeats 13 to 15 is further emphasized by a comparison of the A-subunit structure with the structures of the PP2A core and holoenzymes (5, 46). It seems noteworthy that the N-terminal and C-terminal regions of the A subunit that display conformational flexibility are the same regions that bind reversibly to ST and AR binding regions of PP2A A subunit.

We propose that ST binding to N-terminal HEAT repeats causes a conformational change that is transduced through HEAT repeats 6 to 12, causing a subtle conformational change in the C-terminal HEAT repeats that reveals an AR binding site (Fig. 10A). Because ST is not detected in the AR-PP2A complex (48), AR binding to the C-terminal HEAT repeats in the A subunit may elicit a structural change that is transduced back through HEAT repeats 6 to 12, resulting in a conformational change in the N-terminal HEAT repeats that releases ST. The available data are consistent with the concept that the architecture of the A subunit is uniquely suited for protein binding and for propagating conformational changes from N to C and from C to N (Fig. 10A).

A second important aspect of the PP2A-AR interaction is that a specific androgen-induced conformation of AR is required for the interaction. Androgen-free AR does not bind to PP2A in the ST-dependent transfer reaction, and of all andro-

could generate an AR conformation that is resistant to phosphatase action by limiting phospho-site accessibility. Both kinases and phosphatases have access to ligand-free AR based on the fact that OA treatment results in the accumulation of highly phosphorylated AR. Androgen-dependent phosphorylation of AR could result from contributions from both types of mechanisms.

gen agonists and antagonists tested, only the synthetic androgen R1881, DHT, and ASD were capable of generating an AR conformation compatible with PP2A binding. The requirement for an androgen-bound conformation points to the LBD as the likely binding site for the PP2A A subunit. This view is corroborated by deletion analysis showing that AR lacking the AF-1 or DBD is still functional for PP2A binding, but removal of the LBD abolishes PP2A binding. We also observed a correlation ($R^2 = 0.986$) between dissociation of androgen from AR and PP2A release, evidence that PP2A release occurs when AR returns to the androgen-free conformation.

AR conformation and transcription. The stability of the AR-PP2A interaction enabled us to examine whether AR adopts a conformation that binds PP2A, which the data argue is an androgen-bound conformation, under conditions that are relevant to prostate cancer. The T877A mutation broadens the ligand recognition properties of the AR LBD, and, as a consequence, antagonists including Flut and HO-Flut function as agonists that promote AR-dependent transcription (13, 38, 42). These observations led us to hypothesize that Flut and HO-Flut might, in the context of the T877A mutation, generate the agonist-type conformation of AR. This was not the case, however, as these and other ligands activated AR-dependent transcription of reporter genes and endogenous PSA without generating an apparent androgen-bound conformation of AR. Thus, five different ligands (Flut, HO-Flut, bicalutamide, DHEA, and estradiol) supported T877A AR-dependent transcription without generating the androgen-bound conformation of AR. Our conclusion appears to differ from the conclusion drawn by the Chang and McDonnell (2). Using a mammalian two-hybrid assay to score the interaction between a coactivator peptide (D30) and AR, it was concluded that HO-Flut could, in the context of the T877A mutation, generate the agonist conformation of AR (2). A potential explanation for the difference in interpretation is that PP2A and the D30 coactivator peptide may register different structural features of the antagonist-bound LBD.

The AR-PP2A co-IP assay also allowed us to formally test whether AR adopts an androgen-bound conformation in the presence of castrate levels of androgen. The impetus for this experiment was that castrated mice provide the setting for developing and propagating androgen-independent human prostate tumors. In some prostate cancer models, castrate levels of androgen are insufficient to fully activate AR (50). Our analysis, which was performed in LNCaP cells grown as xenografts in mice, revealed that the conformation of AR that binds PP2A is maintained even after the mice were castrated. This resulted in ST-dependent PP2A transfer to AR and dephosphorylation of phospho-sites in the AF-1. Our data suggest that castrate levels of androgen are sufficient to promote the androgen-bound conformation of AR, though it is possible that signal transduction in the xenograft promotes an AR conformation that binds PP2A through another pathway. We speculate that androgen-sensitive prostate tumor growth might reflect the involvement of an androgen-sensitive component that has a higher dissociation constant for androgen than AR (19).

An alternative view of how ligand can regulate the AR phosphorylation state. Ligand binding increases the phosphorylation state of nuclear receptors, including those that bind steroid hormones. Phospho-site-specific antibodies have been

used to show that phosphorylation levels at sites in the AF-1 region of AR (Ser81, Ser213, Ser256, Ser308, Ser424, and Ser650) (40, 48), GR (Ser203 and Ser211) (43), progesterone receptor (Ser102, Ser 294, and Ser345) (51), and ER (Ser118) (22) increase upon the addition of cognate ligand. The tacit assumption in these studies has been that ligand binding induces a conformational change that exposes AF-1 phosphorylation sites (Fig. 10B, model I). That kinase accessibility to AF-1 sites would be enhanced in response to ligand binding seems reasonable, given the evidence that AF-1 regions of nuclear receptors display ligand-regulated interactions with the LBD (28). According to this view, ligand binding to the C-terminal LBD could promote changes in the N-terminal AF-1 structure that either (i) increase AF-1 solvent exposure, (ii) release proteins that otherwise repress kinase action on the AF-1, or (iii) promote AF-1 kinase recruitment to AR.

Our data suggest an additional mechanism for how the phosphorylation state of the AF-1 region could increase upon ligand treatment (Fig. 10B, model II). We found that treating cells with a concentration of OA that inhibits a subset of phosphatases was sufficient to promote a high level of phosphorylation of five sites in the AF-1 region of AR. Most of these sites in AR undergo robust androgen-dependent phosphorylation. We also observed that phospho-site Ser16, which displays a basal level phosphorylation and is affected only slightly by ligand addition, shows a significantly higher level of phosphorylation in the presence of OA. Our data provide compelling evidence that in cells, these phosphorylation sites are kinase accessible prior to ligand binding. This leads us to propose that the low levels of phosphorylation observed in the AF-1 sites of AR in the absence of androgen are maintained by the constitutive action of one or more phosphatases (Fig. 10B, model II). This could be through the action of phosphatases that are stably bound to AR, phosphatases that interact transiently with AR, or both. According to this model, androgen binding would induce a conformation in AR that is resistant to phosphatase action, either because the phospho-sites become inaccessible to phosphatase or because changes in AR structure are incompatible with efficient phosphatase targeting. We favor the latter scenario since removal of the LBD from AR results in a high level of Ser81 and Ser308 phosphorylation even without OA treatment.

The LBD appears to play a dual role in regulating phosphatase action on AR. In the absence of androgen, the LBD facilitates AF-1 dephosphorylation because the ligand-free form of AR is highly compatible with phosphatase interactions. In the presence of ligand, the LBD appears to repress dephosphorylation either because the ligand-bound form of AR is incompatible with phosphatase interactions or because the ligand-bound form of AR actively represses dephosphorylation. Even though the phospho-sites are kinase accessible prior to androgen binding, the LBD may also make a positive contribution to Ser256 phosphorylation. Ser256 phosphorylation in the Δ LBD mutant of AR was extremely low compared to androgen-bound AR. This could be because the LBD is important, directly or indirectly, for kinase recognition of the Ser256 phospho-site.

The candidates for mediating constitutive dephosphorylation of unliganded AR are the OA-sensitive phosphatases PP5 and PP2A. PP5 is known to bind GR-associated Hsp90, and

through this interaction PP5 can influence GR subcellular localization and transactivation function (9, 11, 39). It is not known, however, whether GR is a direct substrate for PP5-dependent dephosphorylation. By extension, PP5 is predicted to bind AR-associated Hsp90, but to our knowledge this interaction has not yet been demonstrated. Proposing that PP2A is a constitutive phosphatase for ligand-free AR seems at odds with our finding that PP2A binds specifically to the androgen-bound conformation of AR. But it should be noted that this is a special circumstance that occurs in the presence of SV40 ST, and it is correlated with alterations in PP2A A-subunit structure. It is possible that SV40 has coopted a constitutive pathway that normally mediates dephosphorylation of ligand-free AR. ST binding to PP2A shifts the pathway to a dependence on androgen-dependent changes in AR structure and results in androgen-dependent transfer and dephosphorylation of AR. The biological significance of this reaction is not yet clear. It has been shown that combining SV40 ST and large-T-antigen expression in a mouse model of prostate cancer results in a highly metastatic phenotype, whereas SV40 large-T-antigen expression is only tumorigenic (14) (23). Whether ST expression and modulation of AR phosphorylation are causal to some aspect of the metastatic phenotype remains an open question.

In summary, we have shown that protein conformation plays a critical role in determining phosphatase action on AR. Understanding the balance between kinase and phosphatase actions on AR should help explain how extracellular signals are transduced from the plasma membrane to the nucleus and provide the context for defining how alterations in these pathways may contribute to the development of prostate cancer.

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