Androgen Receptor Serine 81 Phosphorylation Mediates Chromatin Binding and Transcriptional Activation*

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Background: Androgen receptor (AR) phosphorylation at serine 81 is stimulated by the mitotic cyclin-dependent kinase 1 (CDK1). In this report, we extended our previous study and confirmed that Ser-81 phosphorylation increases during mitosis, coincident with CDK1 activity. We further showed blocking cell cycle at G1 or S phase did not disrupt androgen-induced Ser-81 phosphorylation and AR-dependent transcription, consistent with a recent report that AR was phosphorylated at Ser-81 and activated by the transcriptional CDK9. To assess the function of Ser-81 phosphorylation in prostate cancer (PCa) cells expressing endogenous AR, we developed a ligand switch strategy using a ligand-binding domain mutation (W741C) that renders AR responsive to the antagonist bicalutamide. An S81A/W741C double mutant AR stably expressed in PCa cells failed to transactivate the endogenous AR-regulated PSA or TMPRSS2 genes. ChIP showed that the S81A mutation prevented ligand-induced AR recruitment to these genes, and cellular fractionation revealed that the S81A mutation globally abrogated chromatin binding. Conversely, the AR fraction rapidly recruited to chromatin after androgen stimulation was highly enriched for Ser-81 phosphorylation. Finally, inhibition of CDK1 and CDK9 decreased AR Ser-81 phosphorylation, chromatin binding, and transcriptional activity. These findings indicate that Ser-81 phosphorylation by CDK9 stabilizes AR chromatin binding for transcription and suggest that CDK1-mediated Ser-81 phosphorylation during mitosis provides a pool of Ser-81 phosphorylation AR that can be readily recruited to chromatin for gene reactivation and may enhance AR activity in PCa.

Results: Chromatin-associated AR is enriched for Ser-81 phosphorylation, and an S81A mutation abrogates AR transcription and chromatin binding.

Conclusion: Ser-81 phosphorylation is required for AR chromatin binding.

Significance: These findings identify a critical function for Ser-81 phosphorylation and a mechanism through which CDK1 may enhance AR activity.

Our previous findings indicated that androgen receptor (AR) phosphorylation at serine 81 is stimulated by the mitotic cyclin-dependent kinase 1 (CDK1). In this report, we extended our previous study and confirmed that Ser-81 phosphorylation increases during mitosis, coincident with CDK1 activity. We further showed blocking cell cycle at G1 or S phase did not disrupt androgen-induced Ser-81 phosphorylation and AR-dependent transcription, consistent with a recent report that AR was phosphorylated at Ser-81 and activated by the transcriptional CDK9. To assess the function of Ser-81 phosphorylation in prostate cancer (PCa) cells expressing endogenous AR, we developed a ligand switch strategy using a ligand-binding domain mutation (W741C) that renders AR responsive to the antagonist bicalutamide. An S81A/W741C double mutant AR stably expressed in PCa cells failed to transactivate the endogenous AR-regulated PSA or TMPRSS2 genes. ChIP showed that the S81A mutation prevented ligand-induced AR recruitment to these genes, and cellular fractionation revealed that the S81A mutation globally abrogated chromatin binding. Conversely, the AR fraction rapidly recruited to chromatin after androgen stimulation was highly enriched for Ser-81 phosphorylation. Finally, inhibition of CDK1 and CDK9 decreased AR Ser-81 phosphorylation, chromatin binding, and transcriptional activity. These findings indicate that Ser-81 phosphorylation by CDK9 stabilizes AR chromatin binding for transcription and suggest that CDK1-mediated Ser-81 phosphorylation during mitosis provides a pool of Ser-81 phosphorylation AR that can be readily recruited to chromatin for gene reactivation and may enhance AR activity in PCa.

Androgens and the androgen receptor (AR) play essential roles in the development and progression of prostate cancer (PCa). AR can be structurally divided into an N-terminal domain (NTD) that harbors a major transcriptional activation function (AF-1), a central DNA-binding domain (DBD), a hinge region that contains a nuclear localization signal, and a C-terminal ligand-binding domain (LBD) that has a minor transcriptional activation function (AF-2) (1). Binding of androgen (testosterone or dihydrotestosterone (DHT)) causes a conformational change in the LBD and generates a hydrophobic cleft that initially binds a hydrophobic helix in the AR NTD (AR N-C interaction) and subsequently mediates binding of LXXLL motifs in transcriptional coactivator proteins. The androgen-ligated AR forms homodimers and accumulates in the nucleus, where it binds to androgen-responsive elements (AREs) in the enhancers of androgen-regulated genes and recruits coactivator proteins through interactions with both the NTD and LBD (2, 3). Androgen deprivation therapy (surgical or medical castration) is used for the initial systemic treatment of PCa, but the disease invariably recurs, and these castration-resistant prostate cancers (CRPCs) are generally more aggressive. Many patients with CRPC will respond to secondary hormonal therapies with AR antagonists or agents that further suppress androgen synthesis, in particular the recently FDA-approved CYP17A1 inhibitor abiraterone, but most of these patients still relapse within a year (4, 5). Significantly, the AR remains highly expressed and transcriptionally active in these advanced tumors, but the molecular mechanisms contributing to this activity at low androgen levels are poorly understood (6).

The abbreviations used are: AR, androgen receptor; PCa, prostate cancer; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; DHT, dihydrotestosterone; ARE, androgen-responsive element; CRPC, castration-resistant prostate cancer; TLB, Triton lysis buffer; CDS, charcoal/dextran-stripped FBS.

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Ser-81 Phosphorylation Is Required for AR Nuclear Functions

AR is phosphorylated constitutively at Ser-94 in the NTD and Ser-650 in the hinge region prior to androgen exposure (7–9). Androgen stimulates AR phosphorylation at multiple additional sites in the NTD, including serines 81, 256, 308, and 424, and increases Ser-650 phosphorylation (9). Recent reports indicate that Ser-650 phosphorylation is stimulated by a stress-induced kinase pathway and enhances AR nuclear export and that this site is dephosphorylated by protein phosphatase 1 (10, 11). However, the functional significance of other androgen-stimulated phosphorylation sites and the kinase pathways regulating phosphorylation of these sites remain to be fully defined (8, 12–15). Previous mass spectrometry studies have shown that Ser-81 is the most highly phosphorylated site in response to androgen stimulation and shown that Ser-81-phosphorylated AR (Ser(P)-81-AR) accumulates gradually over ~8 h in PCa cells after androgen stimulation (9, 16). We have reported that CDK1 activity was increased in CRPC (17) and that CDK1 could phosphorylate AR at Ser-81 and sensitize AR to low levels of androgens (16). A recent study found that, in addition to CDK1, AR was associated with CDK5 and CDK9 and that CDK9 could also phosphorylate AR at Ser-81 (18). In contrast to CDK1, which is activated in mitosis, CDK9 associates with cyclin T to form the P-TEFb complex that stimulates transcriptional elongation through phosphorylation of substrates, including Ser-2 in the RNA polymerase II C-terminal domain (19). Significantly, this latter study showed that an S81A mutant AR was less effective at stimulating growth and, when expressed in AR-negative PCa cells, was decreased in its ability to transactivate a subset of AR-regulated genes (18).

In this report, we first extend our previous studies by establishing that AR Ser-81 phosphorylation is increased during the M phase of the cell cycle and mediated by CDK1 activation. Moreover, by preventing androgen-stimulated PCa cells from progressing to G0/M, we confirmed that Ser-81 also can be phosphorylated independently of CDK1 activation, consistent with the findings showing CDK9-mediated Ser-81 phosphorylation (18). To address the functional significance of Ser-81 phosphorylation in AR-expressing cells, we exploited a W741C mutation in the AR LBD that allows the LBD to fold into the agonistic conformation and AR to be activated by the antagonist bicalutamide (20). Using this ligand switch strategy to stably express and selectively activate a S81A mutant in LNCaP cells expressing endogenous AR, we found that the S81A/W741C double mutant versus the W741C single mutant AR was unable to stimulate endogenous AR-regulated genes in response to bicalutamide. Chromatin immunoprecipitation (ChIP), in conjunction with immunofluorescence and cellular fractionation studies, showed that the molecular basis for this defect was an inability of the S81A mutant to bind stably to chromatin. By ChIP and biochemical analyses, we further found that AR tightly associated with chromatin was enriched for Ser-81 phosphorylation. In addition, both CDK1 and CDK9 antagonists decreased AR Ser-81 phosphorylation, chromatin-binding, and transactivation. Based on these findings, we propose that Ser-81 phosphorylation by CDK9 in non-mitotic cells stabilizes AR binding to chromatin and is required for the subsequent chromatin remodeling and transcriptional activation. Moreover, we suggest that Ser-81 phosphorylation by CDK1 during mitosis provides a pool of Ser(P)-81-AR that can be rapidly recruited to AR-regulated genes when the cells enter G0/G1. Through this mechanism, increased CDK1 activity may contribute to AR activation in CRPC.

EXPERIMENTAL PROCEDURES

Materials—The reagents and sources are as follows. DAPI, propidium iodide, hydroxyurea, and mimosein were from Sigma; the CDK1 inhibitors (CGP74514A and RO-3306) were from Calbiochem; the CDK9 inhibitor (CDK9 inhibitor II) was from Millipore (catalog no. 238811); the protease inhibitor (catalog no. 1861278) and phosphatase inhibitor (catalog no. 1861277) mixtures were from Thermo Scientific; and the micrococcal nuclease (M0247) was from New England Biolabs. The sources for the antibodies and control IgGs were as follows: P-AR-S213 (IMG-561, IMAGE®); P-CDK1-T161 (catalog no. 9114, Cell Signaling); anti-FoxA1 (Ab23738, Abcam); and anti-AR (N20, sc-816), normal mouse IgG (sc-2025), and normal rabbit IgG (sc-2027) (Santa Cruz Biotechnology, Inc.). The protein A (catalog no. 20334) and protein G (catalog no. 20399) were from Pierce, and the SYBR Green PCR mix (catalog no. 4309155) was from Applied Biosystems. Plasmids and providers were as follows: Gal4-AR-C and VP16-AR-N (Lirim Shemeshdini, University of Toledo, Toledo, OH), CDK7 (P#633, Addgene), CDK8 (Dr. Joan Weliky Conaway, Stowers Institute for Medical Research, Kansas City, MO), and CDK9 (Dr. Rosemary Kiernan, Institut de Génétique Humaine UPR1142). Other reagents were described previously (10, 16).

ChIP and FACS Analyses—The ChIP assay was described previously (21). The sequence information for the PCR amplification primers is as follows: PSA enhancer (−4 kb, ARE III) (forward, 5′-GCTTGGATCTGAGAGATATCTC-ATC-3′; reverse, 5′-ACACCTTTTTTTTTCTGGATTTG- TTG-3′); TMPRSS2 enhancer (−14 kb, TMPRSS2-ARE5) (forward, 5′-TGGTCCTGGATGATAGTTT-3′; reverse, 5′-GACATAAGCGCCCCACACAGA-3′); nonspecific chromatin region (irrelevant region on chromosome 18) (forward, 5′-CAGAGGGCTGATCTGGTGAC-3′; reverse, 5′-TGGTCCTGGATGATAGTTT-3′). The primer information for AR-mediated enhancers on the FKBP5 (FKBP5 enhancer) and OPRK1 (OPRK1-ARBS) genes was published recently (22). The FACS assay was described previously (23).

Reporter and Real-time RT-PCR Assays—The luciferase reporter and real-time RT-PCR analyses were described previously (10, 16).

DNA Mutagenesis, Generation of Stable Lines, Immunoblotting, and Immunofluorescence Analyses—These experiments were performed as described previously (10).

Cellular Fractionation Assay—Cellular fractionation analysis using the NE-PER kit was carried out as described by the manufacturer (catalog no. 78835, Pierce), and using the Triton lysis buffer (TLB) (10). For extraction, LNCaP cells grown in a 10-cm dish were washed once in cold PBS, harvested for resuspension in 800 μl of TLB containing protease inhibitor and phosphatase inhibitor but no salt (NaCl), vortexed, and kept on ice for 15 min, followed by centrifugation at 11,000 × g for 5 min. The supernatant was removed and saved, and the pellet was resuspended in 100 μl of TLB containing 50 mM of NaCl.
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

FIGURE 1. CDK1 mediates AR Ser-81 phosphorylation during mitosis. LNCaP cells grown in FBS-containing medium were treated with 50 ng/ml nocodazole (NOCO) for the indicated times. Cells were fixed and stained with propidium iodide (PI) for FACS analysis (A) or harvested in 2% SDS, and total proteins were normalized for Western blotting (B). C, LNCaP cells were treated with nocodazole, together with or without CDK1 inhibitors CGP74514A (CGP; 1 μM) or RO-3306 (RO; 10 μM) for the indicated times. Images of Western blots were quantified for Ser(P)-81/AR ratio using the ImageJ software. P-AR-S81, Ser(P)-81-AR.

To address further whether Ser-81 phosphorylation during mitosis was mediated by CDK1, we challenged nocodazole-treated LNCaP cells with two different CDK1 inhibitors, CGP74514 and RO-3306 (25, 26). Treatment of nocodazole-arrested cells with CDK1 inhibitors led to time-dependent reduction in nocodazole-induced Ser-81 phosphorylation (Fig. 1C). Consistent with our previous findings, the down-regulation of Ser(P)-81-AR by CDK1 inhibition was also associated with reduced levels of AR protein (16). However, quantification of the ratio of Ser(P)-81-AR over total AR indicated that the reduction in Ser-81 phosphorylation was predominant and not solely due to decreased AR protein levels. Together, these findings supported the conclusion that AR Ser-81 is phosphorylated by CDK1 during mitosis.

S or G1 Phase Arrest Does Not Prevent DHT-stimulated AR Ser-81 Phosphorylation and Transcriptional Activation—Previous studies have shown that androgen-induced AR Ser-81 phosphorylation increases progressively and does not reach maximal levels until ~8 h (9, 16). Because androgen treatment also stimulates androgen-deprived PCa cells to cross the G1/S checkpoint and enter the cell cycle (27–29), we considered that the DHT-stimulated increase in Ser-81 phosphorylation could reflect cells progressing to G2/M phase. Therefore, to determine whether the DHT-stimulated increase in Ser(P)-81-AR was dependent on cell cycle progression, we used hydroxyurea to arrest DHT-stimulated LNCaP cells in S phase. Fig. 2A shows that hydroxyurea treatment led to a time-dependent accumulation of cells in S phase. Immunoblotting analysis showed that AR protein, which is increased by hydroxyurea treatment, was decreased by hydroxyurea treatment in the absence and presence of DHT (Fig. 2B). However, DHT still increased Ser(P)-81-AR in the hydroxyurea-treated cells, indicating that Ser-81 phosphorylation is not dependent upon cell cycle progression to M phase. Overall AR transcriptional activity also appeared to be repressed by hydroxyurea, based on immunoblotting for PSA protein (Fig. 2B) and RT-PCR for the AR-regulated PSA and TMPRSS2 genes (Fig. 2C). These decreases in AR expression and activity may reflect S phase arrest or other effects of hydroxyurea,
but the results still indicated that the androgen-stimulated increase in Ser(P)-81 was not dependent on cells reaching M phase.

The observation that AR Ser-81 phosphorylation is not dependent on cell cycle progression to M phase was further verified by treating LNCaP cells with a reversible cell cycle inhibitor, mimosine. This amino acid analog reversibly represses the initiation of DNA replication and synchronizes cells in G1 phase of the cell cycle (30). As shown in Fig. 2D, mimosine treatment had no pronounced effect on DHT-stimulated AR protein expression and transcriptional activity, and, most importantly, Ser-81 phosphorylation. Together these findings demonstrated that Ser-81 can be phosphorylated in response to androgen stimulation independently of cell cycle progression and CDK1 activation and were consistent with the recent study identifying CDK9 as another mediator of Ser-81 phosphorylation (18).

AR Transcriptional Activity in Transient Transfection Assays Is Not Enhanced by CDKs and Is Not Dependent on Ser-81 Phosphorylation—As noted above, a recent study confirmed an association between AR and CDK1 and further showed that CDK9 was associated with AR and could mediate Ser-81 phosphorylation (18). Another transcriptional CDK, CDK7, also has been reported to phosphorylate AR (at Ser-515) and increase AR transcripational activity (31). To assess and compare the effects of these CDKs, we carried out AR-dependent reporter assays in LNCaP cells with co-transfected transcriptional CDKs (CDK7, -8, and -9) or activated CDK1 (CDK1-AF) (16). As shown in Fig. 3A, all tested CDKs failed to enhance the DHT-stimulated activity of AR-regulated ARE4-Luc or PSA-Luc reporter genes in LNCaP cells. We further determined that substitution of Ser-81 by alanine (S81A) did not detectably impair DHT induction of ARE4-Luc or PSA-Luc reporter genes in LNCaP cells, consistent with previous reporter gene studies of this mutant in AR-deficient cells (Fig. 3B) (8, 16). We further examined whether Ser-81 phosphorylation has an impact on the androgen-mediated interaction between the AR NTD and LBD (AR N-C interaction), which is required for AR-mediated transcription and chromatin interaction (33, 34). Using mammalian two-hybrid protein interaction assays with Gal4-AR-LBD and VP16-AR-NTD versus VP16-AR-NTD(S81A), we found that the S81A mutant has comparable N-to-C activity as its wild-type counterpart (Fig. 3C).

Ser-81 Phosphorylation Is Required for Induction of Endogenous Androgen-regulated Genes—Although previous studies and the studies described above have shown that the S81A mutation does not impair the ability of AR to stimulate transiently transfected ARE-regulated reporter genes, it is well...
established that these assays may not effectively reflect the activities of transcription factors on endogenous genes. Indeed, a recent study examining a transfected S81A mutant AR in AR negative PCa cells found a decreased ability to transactivate a subset of endogenous AR regulated genes (18). An approach that has been used to assess the function of a mutant AR in PCa cells expressing endogenous AR has been to express shRNA targeting endogenous AR in conjunction with a stably expressed mutant AR that is not targeted by the shRNA, an approach referred to as the codon switch strategy (35). An alternative approach we employed is based on a well characterized mutation in the AR LBD (W741C) that results in activation by bicalutamide, a clinically used antagonist for the wild-type AR and for the T877A AR mutant expressed by LNCaP cells (20, 36). Importantly, crystal structure analyses of the AR LBD have shown that the bicalutamide-ligated AR W741C mutant folds in the agonist conformation that generates the coactivator binding site, similarly to the androgen-ligated wild-type AR LBD (37, 38). The incorporation of this mutation into an AR that is either wild type or mutated at other sites, which we refer to as a ligand switch strategy, allows us to assess selectively AR mutants by stimulating with bicalutamide without interference from the endogenous AR that is not activated by bicalutamide.

For the ligand switch approach, we generated FLAG epitope-tagged AR expression vectors that were wild type, W741C single mutant, or W741C/S81A or W741C/S81D double mutant (Fig. 4A). Transient transfection with an AR-driven reporter gene (ARE4-Luc) in AR-deficient PC3 cells or AR-expressing LNCaP cells confirmed that bicalutamide could potently activate the W741C mutant AR but not the wild-type AR, whereas

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**FIGURE 4. Ser-81 phosphorylation is required for AR-mediated endogenous but not exogenous promoter activation.** A, schematic showing the ligand switch strategy developed to study AR mutants in PCa cell lines. B–E, PC-3 or LNCaP cells were transfected with 2.5 ng of control CMV-Renilla reporter and 50 ng of ARE4-Luc reporter plasmids, together with 100 ng of FLAG-AR or FLAG-AR-W741C mutant plasmid. Cells in CDS-containing medium were treated for 24 h with either 10 \( \mu \)M bicalutamide (BIC) or 10 nM DHT and assayed for Luc activity. C, LNCaP cells were co-transfected with control and AR-mediated reporters with FLAG-AR-W741C (C), FLAG-AR-W741C-S81A (S81A), or FLAG-AR-W741C-S81D (S81D) expression plasmids in the absence or presence of bicalutamide for 24 h, followed by an assay for Luc activity. D, a two-hybrid reporter assay was carried out similarly in LNCaP cells. ARE4-Luc was co-transfected with AR-DBD-LBD-W741C plasmid, together with VP16-AR-N or Ser-81 mutant expression vectors. Cells were treated for 24 h without or with bicalutamide for the Luc assay. E, LNCaP cells were stably transfected with FLAG-AR-W741C or FLAG-AR-W741C-S81A plasmid. Generated cell lines in CDS-containing medium were treated with 1 or 10 \( \mu \)M bicalutamide or 10 nM DHT for 12 h. Total RNA was isolated for real-time RT-PCR analysis of PSA and TMPRSS2 messages, which are normalized to untreated control. F, LNCaP stable cell lines were treated without or with 10 \( \mu \)M bicalutamide, and whole cell lysates were analyzed by Western blotting. The double arrowheads indicate the transfected FLAG-tagged AR (top) and endogenous AR (bottom) molecules. Error bars, S.D.
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

FIGURE 5. Ser-81 phosphorylation is required for AR chromatin binding activity. A, LNCaP stable lines expressing FLAG-AR were grown in CDS-containing medium for 2 days, treated without or with 10 nM DHT for 2 h, and harvested for ChIP analysis of PSA enhancer using anti-FLAG antibody with mouse IgG as control. B, LNCaP stable cells expressing FLAG-AR-W741C (C) or FLAG-AR-W741C-S81A (S81A) were treated without or with 10 μM bicalutamide for 2 h and harvested for ChIP analysis of PSA enhancer, with a nonspecific chromosome region (NS) as control. C, LNCaP cells were transiently transfected with FLAG-AR-W741C (C) or FLAG-AR-W741C-S81A (S81A) plasmids. Cells were grown in CDS-containing medium, treated without or with 10 μM bicalutamide for 2 h, and harvested for ChIP analysis of PSA and TMPRSS2 enhancers, with the liganded samples normalized to the unliganded ones. D, LNCaP stable cell lines expressing FLAG-AR-W741C (C), FLAG-AR-W741C-S81A (S81A), or FLAG-AR-W741C-S81D (S81D) were treated for 2 h without or with 10 μM bicalutamide and analyzed by ChIP for DNA binding. Error bars, S.D.; *, p < 0.05 in Student’s t test.

both were stimulated by DHT (Fig. 4B). We next tested the S81A/W741C double mutant by transient transfection in LNCaP cells and found that the AR-S81A/W741C and W741C control yielded similar reporter gene activities (ARE4-Luc or PSA control) in both transiently transfected LNCaP cells and found that the AR-S81A/W741C and W741C single mutant controls were stimulated by DHT (Fig. 4B). We next tested the S81A/W741C double mutant by transient transfection in LNCaP cells and found that the AR-S81A/W741C and W741C control yielded similar reporter gene activities (ARE4-Luc or PSA control) in both.

In the W741C single mutation control but not in the double mutant or the endogenous AR. Overall, although the S81A mutant AR is expressed at lower levels, its inability to stimulate PSA or TMPRSS2 gene expression suggested a critical function of Ser-81 phosphorylation in AR-mediated transcription of endogenous but not exogenous genes. 

Ser-81 Phosphorylation Is Required for AR Chromatin Binding—To identify the molecular mechanisms by which Ser-81 phosphorylation may regulate AR transcription, we next carried out ChIP to assess effects of the Ser-81 mutation on binding to AREs in the enhancer locus of endogenous AR-regulated genes. We initially examined an LNCaP stable cell line expressing FLAG-tagged wild-type AR. As shown in Fig. 5A, using anti-FLAG antibody, we confirmed DHT-stimulated binding of the transfected AR to the major ARE in the PSA gene enhancer. Next, we used ChIP to study bicalutamide-stimulated recruitment of the W741C control versus S81A/W741C double mutant ARs in LNCaP stable cells. Bicalutamide induced binding of the W741C control AR to the PSA enhancer but did not increase AR binding to an irrelevant nonspecific (NS) region of chromatin (Fig. 5B). In contrast, there was no detectable recruitment of the S81A/W741C double mutant AR.

We also carried out similar experiments in transiently transfected LNCaP cells and examined the AREs in both the PSA and TMPRSS2 enhancers (TMPRSS2-ARE5) (39). Bicalutamide stimulated binding of the transiently transfected AR-W741C AR to both AREs, although the -fold increase was modest due to transfection of only a subset of cells (Fig. 5C). In contrast, bicalutamide did not stimulate binding of the transiently
AR-S81A/W741C double mutant to either site. Interestingly, bicalutamide caused a slight but consistent decrease in binding of the S81A/W741C mutant AR. This may reflect basal (in the absence of bicalutamide) weak chromatin engagement by the highly overexpressed transfected ARs, which then was decreased by bicalutamide due to competition from bicalutamide-liganded endogenous AR that can associate weakly with AREs (40–42). Finally, we also assessed in LNCaP stable lines the binding of a FLAG-tagged S81D/W741C double mutant AR. ChIP results showed that the S81D mutation, which may simulate Ser-81 phosphorylation, did not prevent AR binding to chromatin and may instead enhance binding (Fig. 5D).

Together, these findings indicated that Ser-81 phosphorylation was required for AR occupancy to AREs in endogenous AR-regulated genes.

Ser-81 Phosphorylation Is Involved in Cellular Distribution of AR—We next performed indirect immunofluorescence to determine whether the defect of the S81A mutation on AR binding to particular AREs was reflected in an effect of Ser-81 phosphorylation on cellular distribution in response to ligand stimulation. It has been well established that in androgen-depleted PCa cells, AR is distributed diffusely in the cytoplasm and nucleus and becomes strongly concentrated in the nucleus in response to androgen (43, 44). Similarly, the FLAG-AR-W741C single mutant in androgen-starved LNCaP stable transfectants was distributed in both the cytoplasm and nucleus, and nuclear expression was enhanced by bicalutamide (Fig. 6A, top). In contrast, the S81A/W741C double mutant AR was almost entirely cytoplasmic in the absence of ligand, and substantial AR remained in the cytoplasm after bicalutamide treatment, with a minor population migrating into the nucleus (Fig. 6A, middle). Finally, the S81D/W741C mutant showed predominant nuclear expression in the absence and presence of ligand (Fig. 6A, bottom).

Although immunofluorescence studies show that wild-type AR in androgen-depleted cells is distributed in both the nucleus...
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

and cytoplasm, it is only weakly associated with the nucleus. Therefore, in cellular fractionation studies, the unliganded wild-type AR is found predominantly in the cytoplasmic fraction, whereas a substantial fraction is recovered in the nuclear fraction in response to androgen stimulation (10). Similarly to the wild-type AR, cellular fractionation studies showed that the FLAG-AR-W741C single mutant in androgen-starved LNCaP cells was recovered primarily in the cytoplasmic fraction (Fig. 6B, top). As expected, bicalutamide decreased the cytoplasmic expression and increased the nuclear recovery of the W741C single mutant AR. The S81D/W741C double mutant behaved similarly to the single mutant, although the nuclear recovery in the absence of ligand was somewhat increased relative to the single mutant (Fig. 6B, bottom). In contrast to these results, only a small fraction of the S81A/W741C double mutant was in the nuclear fraction in the absence of ligand, and this fraction was not increased by bicalutamide (Fig. 6B, middle).

In addition to analyzing the nuclear proteins that are extracted at moderate stringency, we also addressed whether AR was associated with the insoluble fraction that contains histones and other proteins that are tightly bound to chromatin. By boiling the insoluble pellet fractions in SDS to release histones, we found that low levels of the W741C single mutant and W741C/S81D double mutant ARs were associated with this insoluble fraction after bicalutamide stimulation (Fig. 6B). In addition, the W741C/S81D double mutant AR gained enhanced recovery from the insoluble fraction in the absence of ligand, compared with the W741C single mutant counterpart. In contrast, we did not detect any of the W741C/S81A double mutant AR in this fraction, in the absence or presence of ligand.

As a complementary approach to assess nuclear binding, stable cells cultured in complete medium containing steroid hormones (RPMI 1640 with 10% FBS) were lysed in a low salt Triton X-100 lysis buffer, and increasing salt concentrations were used to extract sequentially nuclear proteins. In LNCaP cells expressing the W741C single mutant AR, one pool of AR was released at low salt (0–50 mM NaCl). Additional AR then could be extracted progressively at higher salt concentrations along with the nuclear protein FOXA1, an AR-interacting transcriptional pioneer factor (Fig. 6C, top) (45, 46). The insoluble material that remained after extraction with 600 mM NaCl contained histones and additional AR and FOXA1. The S81D/W741C double mutant AR showed a similar distribution of AR with slightly enhanced nuclear distribution (Fig. 6C, bottom). In contrast, nearly all of S81A/W741C double mutant AR was extracted at low salt (Fig. 6B, middle). These findings, in conjunction with the above immunofluorescence and cellular fractionation analyses, indicate that Ser-81 phosphorylation is not required for nuclear translocation but does enhance AR nuclear retention and is required for stable AR association with chromatin.

Androgen Stimulates Increased Binding of Ser(P)-81-AR to Chromatin—The data described above using the S81A mutant AR indicated that Ser-81 phosphorylation may be required for AR recruitment to AREs in endogenous androgen-regulated genes. ChIP studies of AR binding have shown that androgen stimulates very rapid recruitment of AR, with substantial binding occurring within 15 min (41). In contrast, the increase in Ser-81 phosphorylation in response to androgen is relatively slow, with cellular levels increasing over about 8 h (Fig. 7A), consistent with previous studies (9, 16). Androgen-stimulated accumulation of Ser(P)-81-AR in the nucleus is similarly slow (Fig. 7B), so it was unclear whether these low levels of Ser(P)-81-AR could be contributing substantially to the androgen-stimulated recruitment of AR to AREs. Therefore, to directly address the contribution of Ser-81 phosphorylation in androgen-stimulated AR recruitment to chromatin, we next carried out AR ChIP studies using the Ser(P)-81-AR antibody.

As an initial control to assess the specificity of the Ser(P)-81-specific AR antibody in the ChIP assay, we transfected PC3 cells (an AR-negative PCa cell line) with the S81D mutant AR. This AR in androgen-starved or DHT-stimulated cells was then examined by ChIP for binding to the AREs in the FKBPs or OPRK1 gene loci (the PSA gene is not activated by transfected AR in this cell line) (22). Using an anti-AR antibody directed at the extreme N terminus, we detected DHT-stimulated AR recruitment to both AREs but not to a control nonspecific region (Fig. 7C). In contrast, no binding was detected using the anti-Ser(P)-81-AR antibody in the absence and presence of ligands, indicating that this antibody did not react with non-phosphorylated AR associated with chromatin.

We next stimulated LNCaP cells with DHT for 8 h followed by ChIP with the anti-AR or anti-Ser(P)-81 AR antibody. Quantitative real-time PCR for the ARE in the PSA gene enhancer showed that the Ser(P)-81 antibody precipitated levels of this ARE that were comparable with the levels precipitated by the AR antibody (Fig. 7D, Round 1). Although higher affinity of the Ser(P)-81-AR could make it chromatin-immunoprecipitate more efficiently, this result suggested that a substantial portion of the AR binding to this ARE may be Ser-81-phosphorylated. To further test this hypothesis, we repeated the ChIP on the same lysate using the same antibodies to further deplete the chromatin (Round 2), followed by an additional round of anti-AR ChIP on the depleted lysate (Round 3). Recovery of the ARE by the Ser(P)-81 antibody was decreased in Round 2 (compare lane 6 in Round 1 versus Round 2), indicating that the antibody was at least partially depleting Ser(P)-81-AR-bound chromatin. ARE recovery by the anti-AR antibody in Round 2 was similarly decreased in the lysate that was immunoprecipitated with the AR antibody in Round 1 (compare lane 4 in Round 1 versus Round 2). Immunoprecipitation of this latter anti-AR-depleted lysate for a third time (Round 3) with the same AR antibody showed that ARE recovery was substantially reduced relative to AR ChIP from the control anti-IgG-treated lysate (Round 3, lane 6 versus lane 2), respectively. AR ChIP from the Ser(P)-81-AR antibody–depleted lysate also showed some depletion (Round 3, lane 6 versus lane 2), but it was clear that a large portion of the AR-associated ARE was not depleted by the Ser(P)-81-AR antibody. Although differences in antibody efficiency, possible in vitro AR dephosphorylation during the sequential immunoprecipitations, and other technical factors preclude precise quantitative conclusions, these data indicate that this ARE may be binding substantial levels of both Ser-81-phosphorylated and non-phosphorylated AR.

Previous reports indicated that a high stoichiometric phosphorylation occurred at AR Ser-81 upon 6–8 h of DHT stimu-
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

To further test this hypothesis, we examined the levels of total and Ser-81-phosphorylated AR that were in cytoplasmic and nuclear extracts and that were tightly associated with chromatin at 15 min versus 8 h of DHT stimulation. To more definitively identify the latter tightly chromatin-bound fraction, we used micrococcal nuclease digestion of the insoluble material after nuclear extraction to selectively solubilize proteins that tightly occupy the chromatin. By overexposing the gels, Ser(P)-81-AR could be found prior to DHT treatment in the cytoplasmic fraction, with a very weak band in the nuclear fraction and nothing detectable in the chromatin fraction (Fig. 7F, left). In contrast, upon DHT stimulation for 15 min, Ser(P)-81-AR was increased in the nuclear fraction and was detected in the chromatin fraction (Fig. 7F, middle). The levels of Ser(P)-81-AR in the cytoplasmic and nuclear extracts were much higher after 8 h

FIGURE 7. Dynamics of chromatin-binding and cellular distribution for AR and Ser-81-phosphorylated AR. A, LNCaP cells grown in CDS-containing medium were treated without or with 10 nM DHT for the indicated times. Total proteins were harvested in 2% SDS for Western blotting. B, LNCaP cells grown in CDS-containing medium were treated with 10 nM androgens for the indicated times, and nuclear proteins were isolated using the NE-PER kit for Western blotting. C, PC3 cells transfected of FLAG-AR-S81D were split in CDS-containing medium for 1 day, treated without or with 10 nM DHT for 15 min, followed by ChIP using anti-Ser(P)-81 or anti-AR antibody, with rabbit IgG as control. D, LNCaP cells grown in CDS-containing medium were treated for 8 h without or with 10 nM DHT, followed by sequential ChIP analysis of PSA enhancer ChIP using anti-Ser(P)-81 or anti-AR antibody, with rabbit IgG as control. The depleted supernatant from ChIP Round 1 was applied for ChIP Round 2, with the same antibodies as indicated. The depleted supernatant from ChIP Round 2 was applied for ChIP Round 3, using anti-AR antibody. E, LNCaP cells grown in CDS-containing medium were treated for 15 min without or with 10 nM DHT, followed by ChIP analysis for binding of PSA enhancer by AR versus Ser-81 phosphorylated AR. F, LNCaP cells grown in CDS-containing medium were treated without DHT or with 10 nM DHT for 15 min or 8 h, and cytoplasmic (Ct) and nuclear (Nu) proteins were harvested using the NE-PER kit. The insoluble pellet fraction (INS) was digested without or with micrococcal nuclease (M). Error bars, S.D. P-AR-S81, Ser(P)-81-AR.
but were not substantially further increased in the chromatin fraction (Fig. 7F, right). Finally, comparisons of Ser(P)-81-AR band intensities in the nuclear extracts and chromatin-bound fractions versus total AR in these fractions indicated that the stoichiometry of AR Ser-81 phosphorylation was markedly higher in the chromatin-bound fractions.

These fractionation studies indicated that Ser(P)-81-AR binds tightly to a relatively small number of sites on chromatin and that these sites are rapidly saturated after androgen stimulation. Based on the similar rapid binding of Ser(P)-81-AR to the PSA enhancer ARE, we suggest that these high affinity sites include AREs. However, it was not clear from these data whether AR is phosphorylated prior to chromatin binding or is phosphorylated by CDK9 subsequent to binding.

Inhibition of CDK1 or CDK9 Decreases AR Ser-81 Phosphorylation and Chromatin Binding—We showed previously that CDK1 antagonists could decrease AR protein expression, Ser-81 phosphorylation, and transcriptional activity (16). A recent report showed that CDK9 inhibition with flavopiridol similarly could reduce Ser-81 phosphorylation and AR-dependent transcription of endogenous genes (18). Consistent with these previous data, a CDK1-specific inhibitor (R-3306) decreased levels of basal and DHT-stimulated AR protein and Ser-81 phosphorylation (Fig. 8A). In contrast to the effects of R-3306, a CDK9-specific inhibitor, CDK9 inhibitor II (47) decreased DHT-stimulated Ser-81 phosphorylation without decreasing overall AR protein levels.

To determine whether the decrease in CDK1-mediated basal Ser-81 phosphorylation impaired the initial rapid DHT-stimulated binding of AR to chromatin, we examined LNCaP cells that were pretreated for 8 or 24 h with R-3306. Significantly, the R-3306 pretreatment prevented the rapid AR recruitment to the PSA and TMPRSS2 enhancers after 15 min of DHT stimulation (Fig. 8B). We next determined whether inhibiting the DHT-stimulated Ser-81 phosphorylation mediated by CDK9 would impair AR binding to chromatin. For these studies, androgen-starved LNCaP cells were pretreated for 30 min with CDK9 inhibitor II and then stimulated with DHT for 2 or 8 h. As shown in Fig. 8C, the CDK9 inhibitor impaired AR recruitment to both the PSA and TMPRSS2 enhancers.

Finally, we studied the effects of CDK1 and CDK9 inhibition on AR-mediated transactivation. CDK1 inhibition for 8 or 24 h suppressed the DHT-stimulated increase in PSA and TMPRSS2 mRNA message (Fig. 8D). Pretreatment with the CDK9 inhibitor similarly impaired DHT-stimulated expression of PSA and TMPRSS2, although TMPRSS2 expression appeared to recover...
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

after 8 h (Fig. 8D). Because CDK9 is generally required for transcriptional elongation (19), this apparent recovery after 8 h may instead reflect a decrease in the GAPDH internal control used for normalization. In any case, these results support the conclusion that both CDK1 and CDK9 participate in Ser-81 phosphorylation that is required for AR activity. Based on these findings, we propose that Ser-81 phosphorylation is necessary to achieve stable AR binding and initially activate transcription. This model is further detailed under “Discussion.”

DISCUSSION

The AR undergoes serine phosphorylation at multiple sites in response to ligand binding, with Ser-81 being the most heavily stimulated site and generally correlating with transcriptional activity (9, 16). We reported previously that CDK1 could mediate Ser-81 phosphorylation and enhance the sensitivity of AR to androgens (16). Because CDK1 is activated during mitosis, in this study we first established that AR Ser-81 phosphorylation was increased during mitosis, coincident with CDK1 activation. A more recent report identified an interaction between AR and both CDK1 and CDK9 (a transcriptional CDK) and showed that CDK9 could mediate Ser-81 phosphorylation (18). Consistent with additional kinases, including CDK9, mediating Ser-81 phosphorylation, we found that androgen-stimulated Ser-81 phosphorylation could occur independent of cell cycle progression and CDK1 activation. We also confirmed that inhibition of either CDK1 or CDK9 resulted in decreased Ser-81 phosphorylation. In agreement with previous studies using transiently transfected overexpressed AR mutants and reporter genes (8, 16), we found that the S81A mutation did not interfere with AR stimulation of reporter genes or the AR N-C interaction. To assess the function of Ser(P)-81 in the more physiological setting of PCa cells that are expressing endogenous AR and androgen-regulated genes, we developed a ligand switch strategy by taking advantage of a mutation in the AR-LBD(W741C) that renders the AR responsive to bicalutamide. Significantly, although the AR DBD that prevent DNA binding also impair Ser-81 phosphorylation (15, 48), which suggests that Ser-81 phosphorylation may occur subsequent to DNA binding. It also should be noted that the levels of cellular Ser(P)-81 do not increase rapidly in response to androgen but instead rise progressively over about 8 h, consistent with CDK9 phosphorylation of this site as AR cycles on and off of chromatin (9, 16, 18, 41). In contrast to this slow increase in Ser(P)-81, ChIP studies have shown that androgen-stimulated AR recruitment to chromatin occurs rapidly and is nearly maximal within 15 min. Significantly, despite the low cellular levels of Ser(P)-81 after 15 min of androgen stimulation, we found that a substantial proportion of the AR bound to chromatin was phosphorylated at Ser-81. Nonetheless, immunodepletion experiments carried out after 8 h, when Ser(P)-81 levels were maximal, suggested that a large fraction of AR bound to chromatin was not phosphorylated at Ser-81. To reconcile these results, we suggest that Ser-81 phosphorylation is not required for an initial weak transient interaction with chromatin that is not readily detected by ChIP and that Ser-81 phosphorylation by promoter-associated CDK9 stabilizes this AR binding. We further suggest that this Ser-81 phosphorylation permits the recruitment of additional coactivators and subsequent chromatin remodeling required to initiate transcription. Finally, once chromatin has been remodeled and transcription has been initiated, we suggest that Ser-81 phosphorylation may no longer be critical to maintain transcription.

Because cells are not transcriptionally active during mitosis, there may be a distinct function for CDK1-mediated phosphorylation of Ser-81. Interestingly, recent studies indicate that CDK1 plays an important role in retaining gene expression patterns by phosphorylating EZH2 (49–51) and lineage-specific transcription factors, such as Runx2, that remain associated with chromatin during mitosis (52). Significantly, a previous report suggested that AR also remains associated with mitotic chromatin (53). Based on these findings, we suggest that AR Ser-81 phosphorylation by CDK1 may normally function to mark AR-regulated genes during mitosis, allowing daughter cells to rapidly resume their differentiated functions. Additionally, although AR protein levels have been reported to decline after mitosis (54), CDK1 may generate a pool of Ser-81-phosphorylated AR that can efficiently initiate transcription of androgen-regulated genes. Both of these models are consistent with our previous data indicating that CDK1 can sensitize PCa cells to lower levels of androgen (16) and are supported by our data in this study showing that CDK1 inhibition prevents the rapid DHT-stimulated binding of AR to chromatin. Because CDK1 activity is increased in CRPC (17, 45), this may be one mechanism contributing to the maintenance of AR transcriptional activity. We are currently exploring the therapeutic potential of combined CDK1 inhibition and androgen deprivation therapy.

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REFERENCES

1. Lonergan, P. E., and Tindall, D. J. (2011) Androgen receptor signaling in prostate cancer development and progression. J. Carcinog. 10, 20
2. Shen, H. C., and Coetzee, G. A. (2005) The androgen receptor. Unlocking the secrets of its unique transactivation domain. Vitam. Horm. 71, 301–319
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

3. Centeniera, M. M., Harris, J. M., Tilley, W. D., and Butler, L. M. (2008) The contribution of different androgen receptor domains to receptor dimerization and signaling. Mol. Endocrinol. 22, 2373–2382

4. Yap, T. A., Zivi, A., Omlin, A., and de Bono, J. S. (2011) The changing therapeutic landscape of castration-resistant prostate cancer. Nat. Rev. Clin. Oncol. 8, 597–610

5. Cai, C., and Balk, S. P. (2011) Intratumoral androgen biosynthesis in prostate cancer pathogenesis and response to therapy. Endocr. Relat. Cancer 18, R175–R182

6. Yuan, X., and Balk, S. P. (2009) Mechanisms mediating androgen receptor reactivation after castration. Urol. Oncol. 27, 36–41

7. Wong, H. Y., Burghoorn, J. A., Van Leeuwen, D., Reuter, P. E., Schippers, E., Blok, L. J., Li, K. W., Dekker, H. L., De Jong, L., Trapman, J., Grootegoed, J. A., and Brinkmann, A. O. (2004) Phosphorylation of androgen receptor isoforms. Biochem. J. 383, 267–276

8. Zhou, Z. X., Kempainen, J. A., and Wilson, E. M. (1995) Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol. Endocrinol. 9, 605–615

9. Gioeli, D., Ficarro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, A. D., White, F. M., Christian, R. E., Settlage, R. E., Shabanowitz, J., Hunt, D. F., and Weber, M. J. (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J. Biol. Chem. 277, 29304–29314

10. Chen, S., Kesler, C. T., Paschal, B. M., and Balk, S. P. (2009) Androgen receptor phosphorylation and activity are regulated by an association with protein phosphatase 1. J. Biol. Chem. 284, 25576–25584

11. Gioeli, D., Black, B. E., Gordon, V., Spencer, A., Kesler, C. T., Eblen, S. T., Paschal, B. M., and Weber, M. J. (2006) Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization. Mol. Endocrinol. 20, 503–515

12. van Laar, J. H., Bolt-de Vries, J., Zegers, N. D., Trapman, J., and Brinkmann, A. O. (1999) Androgen receptor heterogeneity and phosphorylation in human LNCaP cells. Biochem. Biophys. Res. Commun. 166, 193–200

13. Wang, L. G., Liu, X. M., Kreis, W., and Budman, D. R. (1999) Phosphorylation/dephosphorylation of androgen receptor as a determinant of androgen agonistic or antagonistic activity. Biochem. Biophys. Res. Commun. 259, 21–28

14. Brinkmann, A. O., Blok, L. J., de Ruiter, P. E., Doesburg, P., Steketee, K., Berrevoets, C. A., and Trapman, J. (1999) Mechanisms of androgen receptor activation and function. J. Steroid Biochem. Mol. Biol. 69, 307–313

15. Jenster, G., de Ruiter, P. E., van der Korput, H. A., Kuiper, G. G., Trapman, J., and Brinkmann, A. O. (1994) Changes in the abundance of androgen receptor isoatypes. Effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. Biochemistry 33, 14064–14072

16. Chen, S., Xu, Y., Yuan, X., Bubley, G. J., and Balk, S. P. (2006) Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. Proc. Natl. Acad. Sci. U.S.A. 103, 15969–15974

17. Stanbrough, M., Bubley, G. J., Ross, K., Golub, T. R., Zhang, Z., Nelson, P. S., Liu, X. S., Brown, M., and Balk, S. P. (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20, 457–471

18. Yuan, X., Li, T., Wang, H., Zhang, T., Barua, M., Bregesi, R. A., Bubley, G. J., Lu, M. L., and Balk, S. P. (2006) Androgen receptor remains critical for cell cycle progression in androgen-independent CWR22 prostate cancer cells. Am. J. Pathol. 169, 682–696

19. Lin, H. K., Hu, Y. C., Yang, L., Altuwajiri, S., Chen, Y. T., Kang, H. Y., and Chang, C. (2003) Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J. Biol. Chem. 278, 50902–50907

20. Vassilev, L. T., Tovar, C., Chen, S., Knezevic, D., Zhao, X., Sun, H., Heimbrook, D. C., and Chen, L. (2006) Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc. Natl. Acad. Sci. U.S.A. 103, 10660–10665

21. Imbach, P., Capraro, H. G., Furet, P., Mett, H., Meyer, T., and Zimmermann, J. (1999) 2,6,9-Trisubstituted purines. Optimization towards highly potent and selective CDK1 inhibitors. Bioorg. Med. Chem. Lett. 9, 91–96

22. Cifuentes, E., Croxen, R., Menon, M., Barrack, E. R., and Reddy, G. P. (2003) Synchronized prostate cancer cells for studying androgen regulated events in cell cycle progression from G1 into S phase. J. Cell. Physiol. 195, 337–345

23. Bai, V. U., Cifuentes, E., Menon, M., Barrack, E. R., and Reddy, G. P. (2005) Androgen receptor regulates Cdc6 in synchronized LNCaP cells progressing from G1 to S phase. J. Cell. Physiol. 204, 381–387

24. Knudsen, K. E., Arden, K. C., and Cavenee, W. K. (1998) Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells. J. Biol. Chem. 273, 20213–20222

25. Zhang, M., Latham, D. E., Delaney, M. A., and Chakravarti, A. (2005) Survivin mediates resistance to antiandrogen therapy in prostate cancer. Oncogene 24, 2474–2482

26. Chynnkwitch, P., Le May, N., Charnneau, P., Compé, E., and Egly, J. M. (2011) The phosphorylation of the androgen receptor by TFIH directs the ubiquitin/proteasome process. EMBO J. 30, 468–479

27. Galbraith, M. D., Donner, A. J., and Espinosa, J. M. (2010) CDK8. A positive regulator of transcription. Transcription 1, 4–12

28. Li, J., Fu, J., Tourouzou, C., Yoon, H. G., and Wong, J. (2006) A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin. Mol. Endocrinol. 20, 776–785

29. He, B., Kempainen, J. A., and Wilson, E. M. (2000) FXL1 and WXXL sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J. Biol. Chem. 275, 22986–22994

30. Guo, Z., Dai, B., Jiang, T., Xu, K., Xie, Y., Kim, O., Nesheiwat, I., Kong, X., Melamed, J., Handratta, V. D., Njar, V. C., Brodie, A. M., Yu, L. R., Veenstra, T. D., Chen, H., and Qiu, Y. (2006) Regulation of androgen receptor activity by tyrosine phosphorylation. Cancer Cell 10, 309–319

31. Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G., Jenster, G., Berrevoets, C., Claassen, E., van Rooij, H. C., Trapman, J., Brinkmann, A. O., and Mulder, E. (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem. Biophys. Res. Commun. 173, 534–540

32. Bisson, W. H., Abagyan, R., and Cavasotto, C. N. (2008) Molecular basis of agonicity and antagonicity in the androgen receptor studied by molecular dynamics simulations. J. Mol. Graph. Model. 27, 452–458

33. Bohl, C. E., Gao, W., Miller, D. D., Bell, C. E., and Dalton, J. T. (2005) Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc. Natl. Acad. Sci. U.S.A. 102, 6201–6206

34. Wang, Q., Li, W., Liu, X. S., Carroll, J. S., Janne, O. A., Keeton, E. K., Chinnaiyan, A. M., Pienta, K. J., and Brown, M. (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol. Cell 27, 380–392

35. Farla, P., Hersmus, R., Trapman, J., and Houtsmuller, A. B. (2005) Antian- drogens prevent stable DNA binding of the androgen receptor. J. Cell Sci. 118, 4187–4198

36. Kang, Z., Pirskanen, A., Jänne, O. A., and Palvimäki, J. J. (2002) Involvement...
Ser-81 Phosphorylation Is Required for AR Nuclear Functions

MARCH 9, 2012 • VOLUME 287 • NUMBER 11
JOURNAL OF BIOLOGICAL CHEMISTRY

8583

of proteasome in the dynamic assembly of the androgen receptor transcription complex. J. Biol. Chem. 277, 48366–48371
42. Masiello, D., Cheng, S., Bubley, G. J., Lu, M. L., and Balk, S. P. (2002) Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J. Biol. Chem. 277, 26321–26326
43. Tyagi, R. K., Lavrovsky, Y., Ahn, S. C., Song, C. S., Chatterjee, B., and Roy, A. K. (2000) Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. Mol. Endocrinol. 14, 1162–1174
44. Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanase, T., Takayanagi, R., and Nawata, H. (2001) The subnuclear three-dimensional image analysis of androgen receptor fused to green fluorescence protein. J. Biol. Chem. 276, 28395–28401
45. Wang, Q., Li, W., Zhang, Y., Yuan, X., Xu, K., Yu, J., Chen, Z., Beroukhim, R., Wang, H., Lupien, M., Wu, T., Regan, M. M., Meyer, C. A., Carroll, J. S., Manrai, A. K., Jänne, O. A., Balk, S. P., Mehra, R., Han, B., Chinnaian, A. M., Rubin, M. A., True, L., Fiorentino, M., Fiore, C., Loda, M., Kantoff, P. W., Liu, X. S., and Brown, M. (2009) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 138, 245–256
46. Gao, N., Zhang, J., Rao, M. A., Case, T. C., Mirosevich, J., Wang, Y., Jin, R., Gupta, A., Rennie, P. S., and Matusik, R. J. (2003) The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. Mol. Endocrinol. 17, 1484–1507
47. Pirngruber, J., Shchebet, A., Schreiber, L., Shema, E., Minsky, N., Chapman, R. D., Eick, D., Aylon, Y., Oren, M., and Johnsen, S. A. (2009) CDK9 directs H2B monoubiquitination and controls replication-dependent histone mRNA 3′-end processing. EMBO Rep. 10, 894–900
48. Black, B. E., Vitto, M. J., Gioeli, D., Spencer, A., Afshar, N., Conaway, M. R., Weber, M. J., and Paschal, B. M. (2004) Transient, ligand-dependent arrest of the androgen receptor in subnuclear foci alters phosphorylation and coactivator interactions. Mol. Endocrinol. 18, 834–850
49. Zeng, X., Chen, S., and Huang, H. (2011) Phosphorylation of EZH2 by CDK1 and CDK2. A possible regulatory mechanism of transmission of the H3K27me3 epigenetic mark through cell divisions. Cell Cycle 10, 579–583
50. Wei, Y., Chen, Y. H., Li, L. Y., Lang, J., Yeh, S. P., Shi, B., Yang, C. C., Yang, J. Y., Lin, C. Y., Lai, C. C., and Hung, M. C. (2011) CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. Nat. Cell Biol. 13, 87–94
51. Chen, S., Bohrer, L. R., Rai, A. N., Pan, Y., Gan, L., Zhou, X., Bagchi, A., Simon, J. A., and Huang, H. (2010) Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. Nat. Cell Biol. 12, 1108–1114
52. Rajgopal, A., Young, D. W., Mujeeb, K. A., Stein, J. L., Lian, J. B., van Wijnen, A. J., and Stein, G. S. (2007) Mitotic control of RUNX2 phosphorylation by both CDK1/cyclin B kinase and PP1/PP2A phosphatase in osteoblastic cells. J. Cell. Biochem. 100, 1509–1517
53. Kumar, S., Chaturvedi, N. K., Kumar, S., and Tyagi, R. K. (2008) Agonist-mediated docking of androgen receptor onto the mitotic chromatin platform discriminates intrinsic mode of action of prostate cancer drugs. Biochim. Biophys. Acta 1783, 59–73
54. Litvinov, I. V., Vander Griend, D. J., Antony, L., Dalrymple, S., De Marzo, A. M., Drake, C. G., and Isaacs, J. T. (2006) Androgen receptor as a licensing factor for DNA replication in androgen-sensitive prostate cancer cells. Proc. Natl. Acad. Sci. U.S.A. 103, 15085–15090