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Mechanisms and Regulation of Gene Expression By Androgen Receptor in Prostate Cancer

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Our general objective is to use completely defined cell-free transcription systems both to identify novel AR-associated cofactors and to investigate the mechanism of action of AR and both novel and previously identified candidate coactivators. In an attempt to isolate cofactors capable of influencing AR transcriptional activity, we used an immunoprecipitation method and identified a 44-kDa protein, designated as p44, as a new AR-interacting protein. P44 interacts with AR in the nucleus and with an androgen-regulated homeobox protein (NKX3.1) in the cytoplasm of LNCaP cells. Transient transfection assay revealed that p44 enhances AR-, glucocorticoid receptor-, and progesterone receptor-dependent transcription but not estrogen receptor- or thyroid hormone receptor-dependent transcription. P44 was recruited onto the promoter of the prostate-specific antigen gene in the presence of the androgen in LNCaP cells. P44 exists as a multiprotein complex in the nucleus of HeLa cells. This complex, but not p44 alone, enhances AR-driven transcription in vitro in a cell-free transcriptional system and contains the protein arginine methyltransferase 5 (PRMT5), which acts synergistically with p44 to enhance AR-driven gene expression in a methyltransferase-independent manner. Our data suggest that a novel mechanism by which the protein arginine methyltransferase is involved in the control of AR-driven transcription. P44 expression is dramatically enhanced in prostate cancer tissue when compared with adjacent benign prostate tissue.
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INTRODUCTION
The androgen receptor mediates the biological functions of androgens in gene expression and is implicated in prostate cancer. Androgen ablation therapy, while effective in early androgen-dependent stages, nonetheless fails in the androgen-independent stages of advanced prostate cancer. Although the mechanism for the clinical response to androgen withdrawal therapy is not clear, androgen-independent progression has been associated with mutations or amplification of the androgen receptor gene and activation of intracellular signal transduction pathways that stimulate the androgen receptor function. Insights into this problem and a possible therapy for prostate cancer may be gained from a detailed understanding of the molecular mechanisms by which androgen receptor activates key target genes in malignant prostate cells. Our hypothesis is (i) that the transition from androgen-dependent to androgen-independent prostatic cancer involves alterations in the levels or activities of coactivators that interact physically and/or functionally with AR to activate genes whose expression contributes to malignancy and (ii) that an understanding of the nature, regulation and mechanism of action of these factors can facilitate the development of anti-prostatic cancer therapy. The general objective is to use completely defined cell-free transcription systems both to identify novel AR-associated cofactors, isolated by conventional fractionation and/or affinity methods, and to investigate the mechanism of action of these and previously identified candidate coactivators. Toward this objective we will (i) investigate purified androgen receptor and cognate cofactor functions in cell-free transcription systems reconstituted with general initiation factors and cofactors, wild type and mutant androgen receptors, and both DNA and chromatin templates, (ii) identify by complementation assays, purify by affinity methods, and characterize mechanistically additional (co)factors that act in association with androgen receptor on androgen receptor-activated genes and (iii) investigate possible changes either in the levels or in the activities (functional modifications) of these factors (e.g. in response to other signal transduction pathways) during prostate cancer development. This approach might provide new drug development insights into targeting the androgen receptor pathway downstream of the point of lig-receptor interaction.
**BODY**

**Task 2** To analyze the cofactor requirements and mechanisms of action of AR on chromatin templates (months 1-12);

- a. purify human core histones from HeLa cells (months 1-6)
- b. express and purify NAP1, ACF1 and ISWI (months 1-6)
- c. assemble chromatin in vitro (months 6-12)
- d. perform transcription assays on the chromatin template (months 6-12)

We have purified histones from HeLa cells, expressed and purified the chromatin assembling factors (NAP1, ACF1, and ISWI), and assembled chromatin in vitro (please see the first annual report for details). We used this system to analyze the roles of p300 and SRC1 in AR-dependent transcription. A template contains the 5S DNA sequences position nucleosomes flanking over the E4 core promoter and upstream 3 AR-binding sites (2, 9). Transcription assays were conducted with the recombinant AR, SRC1, and p300, general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, Pol II), and cofactor PC4 (9). In this system the AR-dependent transcription from the DNA template is independent of added SRC1, p300 and acetyl-CoA (Figure 1, lanes 1-7). Prior assembly of the DNA in chromatin completely repressed the AR-dependent transcription (lane 9).

![DNA template vs Chromatin template](https://example.com/diagram.png)

**Figure 1. Transcription from DNA versus chromatin templates.** The AR activated transcription from the DNA template independently of p300 or acetyl-CoA (lanes 1-7). Assembly of the DNA within chromatin completely inhibited transcription in the presence of AR (lane 9). However, the addition of the combinations of p300, SRC1, acetyl-CoA, and R1881 did not restore the AR-dependent transcription (lanes 10-19).

In contrast to our observations with GAL4-VP16-driven transcription (please see the first annual report for details), the AR-dependent transcription from the chromatin template was not restored even in the presence of SRC1, p300, acetyl-CoA, and androgen (R1881) (lane 19). We have noticed that the signal levels of AR-dependent transcription (lane 2) were much weak than that of Gal4-VP16-driven transcription with the reconstituted system. This may reflect the intrinsic inhibitory domain existing in the AR (3) (APPENDIX I). Assembly of the DNA template into chromatin will significantly decrease the transcription signals in general. Therefore, the higher levels of AR-dependent transcription may be required in order to see the AR-driven transcription with chromatin template.
In order to achieve the higher levels of AR-driven transcription, the future work will be to include various cofactors such as the Mediator (4, 8) and p44-containing (APPENDIX II) complexes into the reconstituted system. The resent study has demonstrated that the ATP-dependent chromatin-remodeling complex (SWI/SNF complex) was required for AR-driven transcription in vivo (5). The SWI/SNF chromatin-remodeling complex will also be tested in the reconstituted transcription system with the chromatin template in the future.

**Task 4** To establish the nontumor prostate and androgen-independent prostate cancer cell lines that stably express a FLAG-tagged androgen receptor (months 13-24):

a. transfect the cell lines MDA PCa 2b and RWPE-1 with pBabe-neo-FLAG:AR (months 13-18)

b. select the drug resistant cell line (months 13-18)

c. analyze the drug resistant cell lines (months 19-24)

BPH (non-neoplastic) and PC3 (androgen-independent tumorigenic) prostate cell lines are wildly used and are available from the American Type Cell Culture (ATCC) (6). They grow well in the RPMI1640 medium in tissue culture. In contrast, RWPE-1 (non-neoplastic) and MDA PCa2b (androgen-independent tumorigenic) are not commonly used and need special medium for their growth in the tissue culture (1,7). Therefore, we have chosen BPH and PC3 cells instead of RWPE-1 and MDA PCa2b cells for the proposed studies. BPH and PC3 cell lines were transfected with pBabe-Neo-FLAG:AR and selected with G418. The G418 resistant cell lines were analyzed for the expression of the FLAG-tagged AR by Western blot analysis with anti-AR antibody. Two PC3 cell lines (PC3-AR-2 and PC3-AR-9) express the high levels of AR (Figure 2, lanes 3 and 2). Consistent with the published observations (10), we found that the PC3 cells expressing ectopic AR grow slower and have the higher apoptotic rate than the parent PC3 cells (data not shown). At the same time and under the same conditions, we did not obtained any BPH cell line expressing the ectopic FLAG-tagged AR. This indicated that AR might inhibit the growth of BPH cells. This is consistent with our resent observations that the expression of AR in the primary prostate epithelial cells was lost after growing in the tissue culture for two weeks (data not shown). Currently, we are using the Tet-inducible system (Clonetech) to express the FLAG-tagged AR in BPH cells. The AR-containing complexes will be immunopurified from cytoplasmic or nuclear extracts made from these cell lines growing in the presence or in the absence of the synthetic androgen R1881.
Task 5  **Purification and peptide sequence analysis of f:AR-associated proteins (months 13-24):**

a. grow f:AR-LNCaP cell line (months 13-24)

b. make nuclear and cytoplasmic extracts from f:AR-LNCaP cells (months 13-24)

c. analyze sequences of the f:AR-associated polypeptides (months 18-24)

The large amounts of the f:AR-containing complexes were purified from the nuclear extracts made from the f:AR-LNCaP cells. The AR-associated polypeptide (44 kDa) was submitted for peptide sequence analysis by mass spectrometric techniques (please see APPENDIX II for details).

Task 6  **Cognate cDNA cloning, recombinant protein expression and antibody production (months 25-36):**

a. clone cognate cDNA of the f:AR-associated polypeptides (months 25-30)

b. express and purify the f:AR-associated polypeptides (months 25-30)

c. make polyclonal antibodies against the f:AR-associated polypeptides (months 25-30)

Based on peptide sequence information we have cloned the cognate cDNA encoding a 44-kDa protein. The recombinant protein (p44) was expressed in and purified from bacteria. The antibody against this protein has been produced for a variety of physical and functional studies (please see APPENDIX II for details).

Task 7  **Functional analysis of f:AR-associated proteins (months 30-36):**

a. perform in vitro transcription assays with the f:AR-associated polypeptides (months 30-36)

b. perform western blotting analysis of the f:AR-associated polypeptides (months 30-36)

c. perform in vivo transient transfection analysis with the f:AR-associated polypeptides (months 30-36)

P44 interacts with AR in the nucleus and with an androgen-regulated homeobox protein (NKX3.1) in the cytoplasm of LNCaP cells. Transient transfection assay revealed that p44 enhances AR-, glucocorticoid receptor-, and progesterone receptor-dependent transcription but not estrogen receptor- or thyroid hormone receptor-dependent transcription. P44 was recruited onto the promoter of the prostate-specific antigen gene in the presence of the androgen in LNCaP cells. P44 exists as a multiprotein complex in the nucleus of HeLa cells. This complex, but not p44 alone, enhances AR-driven transcription in vitro in a cell-free transcriptional system and contains the protein arginine methyltransferase 5 (PRMT5), which acts synergistically with p44 to enhance AR-driven gene expression in a methyltransferase-independent manner. Our data suggest that a
novel mechanism by which the protein arginine methyltransferase is involved in the control of AR-driven transcription (please see APPENDIX II for details).

**Task 8** Cell specificity and function of fAR-associated cofactors (months 30-36):

a. perform Western blotting analysis (months 30-36)

b. perform in situ hybridization with prostate cancer samples (months 30-36)

Related to Task 8, we studied the p44 expression by quantitative in situ RNA hybridization in 44 primary prostate cancers with different degree of differentiation. Our results revealed that p44 expression is dramatically enhanced in prostate cancer tissue when compared with adjacent benign prostate tissue (please see APPENDIX II for details).
KEY RESEARCH ACCOMPLISHMENTS
1. Purification, identification and cloning of a novel cofactor (p44) that enhances the AR-dependent transcription both in vitro and in vivo.
2. Found the enhanced expression of p44 in most of the primary prostate cancer tissues, indicating that p44 may play important roles in prostate tumorigenesis.

REPORTABLE OUTCOMES
A manuscript (Appendix II) has been accepted for publication in Molecular and cellular Biology.

CONCLUSIONS
We still have some problems related to task 2 (transcription with the chromatin template) and have partially finished task 4. We have focused our efforts on the purification, identification, and cloning of the AR-associated polypeptides (tasks 5 to 7) during the last year (July 1, 2002 to June 30, 2003). The results obtained from these studies have been included in the paper published on Molecular and Cellular Biology (Appendix II). Further studies will be focused on tasks 2 and 4 and on the mechanism of p44 action in prostate tumorigenesis and prostate cancer growth.
REFERENCES


Identification of a Highly Conserved Domain in the Androgen Receptor That Suppresses the DNA-binding Domain-DNA Interactions*

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The androgen receptor (AR) is a ligand-regulated and sequence-specific transcription factor that activates or represses expression of target genes. Here, we show that the N terminus of AR contains an inhibitory domain located in an 81-amino acid segment lying upstream of the DNA-binding domain (DBD). The inhibitory domain interacted directly with DBD and repressed DBD binding to the androgen response element. Mutations of the conserved amino acid residues K520E and R538E within the inhibitory domain decreased its inhibiting ability in vitro and increased AR trans-activation in vivo. These data demonstrate the existence of a novel inhibitory domain in the N-terminal part of AR, which might play important roles in the regulation of AR trans-activation.

The androgen receptor (AR) mediates androgen functions in the differentiation and maturation of the male reproductive organs and in the development of male secondary sex characteristics. Mutations in the AR gene are associated with the androgen insensitivity syndrome. Numerous somatic mutations in the AR gene have been reported among prostate cancer patients and as well as in prostate cancer cell lines and xenografts. Most of these mutations have been detected in tumor tissues of late-stage prostate carcinoma, indicating that somatic mutation of the AR gene might be involved in the progression and aggressiveness of prostate cancer. The AR is a member of the nuclear receptor superfamily. These receptors are characterized by distinct functional domains: an N-terminal part involved in ligand-independent transcription activation (AF1), a DNA-binding domain (DBD), and a C-terminal ligand-binding domain involved in ligand binding and ligand-dependent transcription activation (AF2). As for other steroid receptors, ligand binding is generally believed to result in a conformational charge in AR with consequent dissociation of heat shock proteins/chaperones, dimerization, and binding to cognate androgen response elements (AREs) in target genes and (through its AF1 and AF2 domains) interactions with various corepressors that facilitate transcription by the general transcriptional machinery. The DBD encompasses two zinc-finger-like modules and binds as dimers to two hexameric sequences orientated as direct or inverted repeats. Although the DBD and the ligand-binding domain of steroid hormone receptors are highly conserved, there is much less homology among steroid hormone receptors in their N-terminal parts. The AR has a long N-terminal part with a strong autonomous AF1 and interacts directly with AF2 in the C-terminal part. The N- and C-terminal interactions are important for androgen-induced gene regulation, and disruption of these interactions may be linked to androgen insensitivity syndrome. The conserved FXXLF and WXXLF motifs within the N-terminal part seem to be involved in pairwise interactions between AF1 and AF2. The N-terminal part contains stretches of glutamates (coded by CAG) and glycine (coded by GGN). Expansion of the CAG repeats is associated with X-linked spinal and bulbar muscular atrophy. A shorter CAG repeat is associated with an increased trans-activation of AR, but the biological role of GGN repeats is less clear.

In this study, we demonstrated that AR contains a highly conserved inhibitory domain within the N-terminal region. The inhibitory domain interacted directly with DBD and inhibited the DBD-DNA interactions. The mutations in the inhibitory domain resulted in decreased inhibitory ability and increased AR trans-activation activity, indicating that this domain might play important roles in the regulation of AR function.

EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant Proteins—The human full-length AR was expressed in SF9 cells via the baculovirus expression vector pVL1393 (BD Biosciences), and the recombinant AR was purified as described previously (20). All of the AR and glucocorticoid receptor (GR) cDNA fragments were amplified by PCR with specific oligonucleotides, cut with NdeI and BamHI, and subsequently cloned in the corresponding restriction sites of the vectors pET15d (Novagen), pGEX-2TL (Amersham Biosciences), and pcDNA3.1 (Invitrogen). The PCR fragments were expressed as His-tagged (via pET15d) or GST fusion (via pGEX-2TL) proteins in Escherichia coli BL21 and purified through nitrolotric acid Ni²⁺-agarose or glutathione-Sepharose columns, respectively. Point mutations were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions and confirmed by DNA sequencing analysis. The mutated proteins were expressed and purified similarly.

Gel Shift Assay—Two pairs of oligomers (5'-AGCTTTTGCAGAACAAGCAATGGTGCACGGG-3' and 5'-AAATTCTAGCTGAGCTGTGGCCTGGGCAAAA-3'; 5'-AGCTTTTGCAGAATACTAGCTGTGGCCTGGGCAAAA-3' and 5'-AAATTCTAGCTGAGCTGTGGCCTGGGCAAAA-3') derived from the prostate-specific antigen gene (−152 to −174) were synthesized, annealed, and subcloned into HindIII and EcoRI sites of the vector pBlue.

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# The abbreviations used are: AR, androgen receptor; AF, transcription activation; DBD, DNA-binding domain; ARE, androgen response element; GR, glucocorticoid receptor; GST, glutathione S-transferase; Luc, luciferase; ID, inhibitory domain.
Identification of an Inhibition Domain of AR

The underlined bases were mutated from their corresponding bases in the wild-type prostate-specific antigen gene sequence. The wild-type and mutant ARE probes were made by cutting these constructs with XhoI and XbaI and purification of fragments from agarose gel. Probes were labeled with [γ-32P]dCTP by a fill-in reaction with the Klenow enzyme. In gel shift assays, 20 µl reaction contains 20 µM HEPES, pH 7.9, 70 mM KCl, 1 µg of poly(dI-dC), 1 mM dithiothreitol, 0.1% Nonidet P-40, 100 µg/ml of bovine serum albumin, and various proteins. The reaction mixture was incubated for 20 min at room temperature, and the binding reaction was initiated by the addition of the labeled probes (20,000 cpm) and then incubated for an additional 30 min at room temperature. The reaction mixture was loaded directly onto a 4% (37.5:1, acrylamide:bisacrylamide) nondenaturing polyacrylamide gel with 0.25 × Tris borate EDTA and run at 150 V for 2 h at room temperature.

Cell Culture and DNA Transfection—PC3 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Cells (5 × 10^6) were plated in each well of 24-well plates and transfected with 100 ng of 4×ARE-E4-Luc reporter plasmid, 2.5 ng of control plasmid pRL-CMV, and various amounts of expression plasmids. Cells were grown in the presence of 10 µM R1881 for 48 h after transfection and harvested for dual-luciferase activity assay (Promega).

Protein-Protein Full-down Assay—GST and GST-DBD (AR537-644) were expressed in bacteria and immobilized on glutathione-Sepharose beads. Beads (10 µl) containing 100 ng of GST or GST-DBD proteins were incubated with 5 µl of transcription and translation coupled rabbit reticulocyte lysates containing 35S-labeled AR477-538 in BC150, 0.1% Nonidet P-40 for 2 h at 4°C. After being washed with the incubation buffer, beads were boiled with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography.

RESULTS

The Full-length AR Interacts with the Androgen Response Element More Weakly than the DBD. A, diagram of domains and truncations of AR. B, SDS-PAGE analysis of the recombinant AR and DBD. Proteins of 100 ng (lane 2) and 200 ng (lane 3) of the purified recombinant AR expressed in Sf9 cells and recombinant His-tagged truncations of AR expressed in bacteria (lanes 4–6) were subjected to SDS-PAGE with Coomassie Blue R250 staining. Lane 1 is standard protein markers (Bio-Rad). C, the gel shift assay was performed using a DNA probe containing the wild-type ARE (lanes 1–5) or the mutant ARE (lanes 6–10). 0.9 pmol of AR (lanes 2, 3, 7, and 8) or 0.6 pmol (lanes 4 and 9) or 0.6 pmol (lanes 5 and 10) of AR537-644 were used in the binding reactions. The synthetic androgen R1881 (100 nm) was included in the reactions in lanes 3 and 8, and lanes 1 and 6 are probes only. D, sequences of the wild-type (AREwt) and mutant (AREmt) ARE and the ARE consensus. The mutated bases in AREmt are underlined. PSA, prostate-specific antigen. M, standard molecular markers. Qn and Qh, stretches of glutamines and glycines, respectively. LBD, ligand-binding domain. AR+L, the gel shift assay performed in the presence of androgen R1881.

A Domain within the AR N Terminus Inhibits DBD-ARE Interactions—A C-terminal extension of the DBD of AR was found to be required for specific and high affinity interactions of DBD with ARE (25). To investigate whether the sequences surrounding DBD would affect DBD-DBD interactions, AR537–662 and AR477–644 (Fig. 1A) were expressed in and purified from bacteria (Fig. 1B, lanes 5 and 6). AR537–662 strongly interacted with the probe, similar to AR537–644 (Fig. 1A, lanes 5–8). However, AR477–644 completely lost the ability to interact with the ARE probe even though much more protein (up to 1.6 pmol) was used in the binding reaction (lanes 5–7). The N-terminal extension of DBD (amino acid residues 477–558) was expressed and purified (Fig. 2B, lane 1). Its molecular mass as determined by SDS-PAGE (16 kDa) is much bigger than the calculated mass (10 kDa), and it was heavily degraded (Fig. 2B, lane 1). This region contains 20% charged amino acids and 16% proline residues, which may be responsible for this aberrant mobility of the protein. When AR477–558 was added to the binding reaction that contained the fixed amount (0.3 pmol) of AR537–644, the density of the DBD-ARE complex dramatically decreased (Fig. 2C, lanes 5–8). These results indicate that AR477–538 specifically inhibits the DBD-ARE interactions in trans as well as in cis. We noticed that different preparations of AR477–538 contained various amounts of the full-length protein and that amounts of the full-length protein (Fig. 2B, lane 1, indicated by the top arrow on the right) were correlated with the inhibition ability of AR477–538. As negative controls, the recombinant prostate apoptosis response-4 (26), 30-kDa Tat-interaction protein (27), and 39-kDa subunit of RNA polymerase C (28) expressed and
Identification of an Inhibition Domain of AR

**The Inhibitory Domain Interacts with DBD and Inhibits AR Trans-activation**—The protein-protein pull-down assay was performed to investigate whether the inhibitory domain (ID) interacts directly with DBD. GST and GST-DBD (AR537-644) fusion protein were expressed in bacteria and immobilized on glutathione-Sepharose beads (Fig. 3A, lanes 2 and 3). The immobilized GST-DBD pulled down 35S-labeled AR477-558 in vitro. The GST and GST-DBD fusion protein are indicated by arrows on the right. The ID inhibited AR trans-activation most likely by blocking the interaction of the AR with the ARE. The lower band (Fig. 3A, lane 2) was not significantly affected by the ID. The ID bound to GST-DBD (lane 3) and not to GST (lane 5). This result indicates that the inhibitory domain interacts directly with DBD.

**Mutations in the ID Enhance AR Trans-activation**—Sequence alignment shows that the ID of AR is highly conserved through evolution (Fig. 5A). To further characterize the biolog-
Identification of an Inhibition Domain of AR

A, sequence alignment of DBD and ID of AR with the corresponding regions of rat GR. B, SDS-PAGE analysis of the recombinant GR418-525 and GR357-525. 500 ng of His-tagged GR418-525 (lanes 1) and GR 357-525 (lane 2) expressed in bacteria were subjected to SDS-PAGE with Coomassie Blue R250 staining. The bands corresponding to the full-length protein fragments are indicated by arrows on the right, and a nonspecific background band is marked by a star on the left. The standard protein markers (Bio-Rad) are indicated on the left. C, GR417-525 and GR357-525 bind to the ARE. 0.3 pmol of GR417-525 (lane 2) or GR515-525 (lane 3) was used in the binding reactions. Lane 1 is the probe-only control.

The gel shift assay revealed that mutations of K520E and R538E decreased the inhibitory ability of ID on DBD-ARE interaction only 65 and 35%, respectively (lanes 9 and 13), or with wild-type (lanes 2, 6, 10, and 14), or with 0.005 pmol (lanes 8, 12, and 16) of wild-type (lanes 8–12). K520E mutant (lanes 9–12) or R538E mutant AR477-558 (lanes 13–16). WT, wild type.

DISCUSSION

The N-terminal parts of nuclear receptors are the most divergent among members of this superfamily of proteins, suggesting that each receptor will take on a unique N-terminal conformation to determine its specificity. This paper describes a highly conserved novel inhibitory domain designated ID, which lies in N-terminal 81-amino acid residues upstream of the DBD of AR. ID interacts directly with DBD and strongly inhibits the DBD-ARE interactions in vitro and AR transactivation in vivo.

Much of the work devoted to understanding regulation of transcription by the AR has focused on the N-terminal AF1 and the C-terminal AF2 (30). However, transcriptional inhibition may be equally important as a way of preventing activation. Studies that deal with inhibition of AR-dependent transcription have focused on silencing mechanisms through recruitment of corepressors to the target promoters and through receptor occupancy at one DNA site interfering with transcription by an activator at an adjoining site (5, 31). We have now demonstrated that negative function element exists in the AR molecule itself and markedly suppresses the DNA binding activity of DBD. The ID function is similar to that of the C-terminal AF1, which lies in N-terminal 81-amino acid residues upstream of the DBD of AR. ID interacts directly with DBD and strongly inhibits the DBD-ARE interactions in vitro and AR transactivation in vivo.

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DNA-binding form as a result of the C-terminal tail-DNA-binding domain interactions (33, 34). Phosphorylation of lysine residues in the C-terminal region leads to the disruption of binding domain interactions (33, 34). Phosphorylation of lysine DNA-binding form as a result of the C-terminal tail-DNA-binding activity of the protein fragment containing the DBD in indicated that the unknown protein could enhance the DNA regulate the DNA binding activity of AE. A study on the rat AR in response to dihydrotestosterone, and these mutations bhibit 2-4-fold increased activity compared with the wild-type AR function, and dysfunction of ID may contribute to prostate cancer or androgen insensitivity syndrome in some men. However, the molecular mechanism for this phenomenon is unclear.

Several mutations found in men with prostate cancer (37) and in men with the androgen insensitivity syndrome (38, 39) localize in ID (Fig. 5A). These mutations might change the function of ID, therefore affecting AR trans-activation. D528G mutation was detected in a patient with prostate cancer (37), and we found that AR with D528G mutation was more active (>3-fold) than the wild-type AR in transient transfection assays (data not shown). Currently, we are investigating whether the enhanced AR trans-activation is because of the decreased ID function. Thus, ID may play an important regulatory role in AR function, and dysfunction of ID may contribute to prostate cancer or androgen insensitivity syndrome in some men.

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REFERENCES
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Enclosure
APPENDIX II

Zhengxin Wang

Hosohata et al.

Purification and identification of a Novel Complex Which Is Involved in Androgen Receptor-Dependent Transcription

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Running title: Identification of a novel androgen receptor cofactor complex

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The androgen receptor (AR) binds to and activates transcription of target genes in response to androgens. In an attempt to isolate cofactors capable of influencing AR transcriptional activity, we used an immunoprecipitation method and identified a 44-kDa protein, designated as p44, as a new AR-interacting protein. P44 interacts with AR in the nucleus and with an androgen-regulated homeobox protein (NKX3.1) in the cytoplasm of LNCaP cells. Transient transfection assay revealed that p44 enhances AR-, glucocorticoid receptor-, and progesterone receptor-dependent transcription but not estrogen receptor- or thyroid hormone receptor-dependent transcription. P44 was recruited onto the promoter of the prostate-specific antigen gene in the presence of the androgen in LNCaP cells. P44 exists as a multiprotein complex in the nucleus of HeLa cells. This complex, but not p44 alone, enhances AR-driven transcription in vitro in a cell-free transcriptional system and contains the protein arginine methyltransferase 5 (PRMT5), which acts synergistically with p44 to enhance AR-driven gene expression in a methyltransferase-independent manner. Our data suggest that a novel mechanism by which the protein arginine methyltransferase is involved in the control of AR-driven transcription. P44 expression is dramatically enhanced in prostate cancer tissue when compared with adjacent benign prostate tissue.
INTRODUCTION

The androgen receptor (AR) mediates androgen function in the development and maintenance of normal prostate tissue (4). The growth and progression of prostate cancer is also dependent on AR. AR is a member of the nuclear receptor superfamily and, like other members of this family, contains a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) with an associated activation function (AF-2) activation domain, and an N-terminal domain (NTD) containing the AF-1 activation domain (9, 20). On ligand binding, AR dissociates with heat shock proteins and chaperones, dimerizes and binds to cognate androgen response elements (AREs) in target genes, and, through its AF-1 and AF-2 domains, interacts with various coactivators that facilitate transcription by the general transcriptional machinery (9, 20). As demonstrated in studies of other activators, gene activation by AR is thought to require the general initiation factors that form pre-initiation complexes on common core promoter elements (e.g. TATA) (45) and a variety of general and gene-specific coactivators that either modulate chromatin structure (26, 36) or serve as direct adaptors between activators and general initiation factors (44). A variety of cofactors have been implicated more directly in nuclear receptor function (32, 33, 55). On a growing list of cofactors that regulate nuclear receptors are the well-studied coactivators of p300/CBP, the p160 family (SRC-1, TIF-2/GRIP-1, ACTR/P-CIP) (55), p300/cREB-binding protein associated factor/GCN5 complexes (yeast SAGA, human STAGA) (5, 31) and protein arginine methyltransferases (PRMTs) (49). These cofactors have histone acetyl transferase or PRMT activities and are believed to act mainly through histone acetylation or methylation and subsequent chromatin structural perturbations but can also act through functional modification of activators (21) and coactivators (10, 56). Some exhibit ligand-dependent interactions with
the AF-2 domain of receptors, whereas others interact with the AF-1 domains. The multiprotein thyroid hormone receptor-associated protein (TRAP)/Mediator complexes exhibit no intrinsic histone acetyltransferase activity (30) and show subunit-specific interactions with both nuclear receptors (TRAP220 with thyroid hormone receptor [TR], vitamin D receptor, peroxisome proliferator-activated receptor, retinoic acid receptor, retinoid X receptor, and estrogen receptor [ER] and TRAP170/vitamin D receptor-interacting protein 150 with glucocorticoid receptor [GR]) and other activators (TRAP80 with p53 and VP16). This complex, in turn, interacts with the general initiation factors and Pol II and acts on DNA templates at postchromatin-remodeling steps. Of these various coactivators, p300/CBP and p160s have been shown to function with AR (1, 3, 6, 23, 29), but functions of the multicomponent STAGA (~15 subunits) and TRAP (~25 subunits) complexes with AR are also likely (52). Other cofactors that have been implicated in the function of AR and, in most cases, other nuclear receptors include the ARA group, ARIP3, SNURF, FHL2, cyclin D1, and AES (22, 24). Some of these factors have broader effects on basal transcription and other activators, but less is known about their mechanistic function.

Homeoboxes are conserved 61-amino acid DNA-binding domains present in a distinct family of transcription factors, homeodomain proteins, that play a central role in eukaryotic development, with spatial and temporal specificity (19). Consistent with their role in cell growth and differentiation, homeobox gene dysfunctions have been implicated in tumorigenesis (12). NKX3.1 is a newly discovered prostate tissue-specific and androgen-regulated gene in the homeobox gene family (42). NKX3.1 is most closely related, by virtue of 78% sequence similarity with the homeodomain region, to Drosophila NK-3. NK-3 interacts with the corepressor Groucho through the homeodomain region to repress transcription (11). Consistent
with its sequence similarity to NK-3, NKX3.1 has been shown to specifically repress transcription of a luciferase reporter containing three copies of the NKX3.1-binding site upstream of a thymidine kinase core promoter (50). The chromosomal association of the NKX3.1 gene on 8p21, a region frequently deleted in prostate cancer, suggests that NKX3.1 may function as a tumor suppressor (8). Consistent with these findings, the results of recent studies of NKX3.1 knockout mice suggest that NKX3.1 exerts a growth-suppressive effect on prostate epithelial cells and controls differentiated glandular functions (2, 7, 25, 46). These findings suggest that, as a transcription factor, NKX3.1 may play an important role in prostate cell development, cell differentiation, and tumorigenesis, even though the biological and biochemical functions of NKX3.1 remain to be deciphered.

In this study, we have identified a new AR-associated protein (p44) that interacts with AR directly and enhances AR-driven gene expression in vivo. We also demonstrated that in the nucleus of HeLa cells, p44 forms a multiprotein complex that functions as a coactivator of AR.
MATERIALS AND METHODS

Establishment of prostate cell lines that stably expressed a FLAG-tagged AR or NKX3.1 and immunopurification of f:AR- and f:NKX3.1-associated factors. The mammalian expression vectors pBabe-Neo-f:AR and pBabe-Neo-f:NKX3.1 were created by subcloning FLAG-tagged human AR or NKX3.1 cDNA into vector pBabe-Neo. The prostate cancer cell line LNCaP was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium plus 10% fetal bovine serum. Cells were transfected with pBabe-Neo-f:AR or pBabe-Neo-f:NKX3.1 and further incubated at 37 °C for 1-1.5 days before being split 1:6 for G418 selection (0.5 mg/ml). The medium was changed every 3 or 4 days. Individual G418-resistant colonies, normally seen after two weeks, were expanded into cell lines and then characterized by Western blotting using the anti-FLAG M2 monoclonal antibody. The cell lines expressing FLAG tagged-AR or FLAG tagged-NKX3.1 were further expanded and analyzed. Nuclear and cytoplasmic extracts were prepared according to our standard methods (54) and used to immunopurify the AR- or NKX3.1-containing complexes. Typically, 1ml of nuclear or cytoplasm extract was mixed with 20 μl of M2 resin and incubated for 3 h at 4 °C with rotation. Washing five times in a buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 150 mM KCl, and 0.1% NP40, the bound proteins were eluted from the M2 agarose by incubation at 4 °C for 30 min with 20 μl of the same buffer plus 0.2 mg/ml of the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys). 5 μl of aliquots of eluted proteins were mixed with equal volumes of 2x Laemmli's sample buffer and loaded onto a 10% polyacrylamide-SDS gel. Proteins were visualized by silver staining.
In vitro transcription and primer extension. The basal transcription factors TFIIA, TFIIIB, TFIIE, and TFIIIF and PC4 were expressed in and purified from bacteria. TFIID, TFIIH and RNA polymerase II were affinity purified from the stable cells expressing corresponding FLAG-tagged subunits (57). Transcription reactions were carried out in a final volume of 25 µl and contained 90 fmol of supercoiled plasmid DNA template; the products were analyzed by the primer extension reaction as described previously (57).

cDNA cloning and Northern blot analysis. An immunopurified f:NKX3.1-containing complex was subjected to SDS-PAGE; and peptides derived from p44 were subjected to mass spectrometric analysis (37). An Expression Sequence Tag (EST) clone (IMAGE:785275) encoding full-length p44 was obtained from American Type Culture Collection (ATCC) (Manassas, VA). A 1.3-kb cDNA encoding full-length p44 was labeled with 32P using the Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH) and was used to probe human multiple tissue Northern blot membranes (BD Biosciences Clontech, Polo Alto, CA).

Expression and Purification of recombinant proteins and antibody preparation. Recombinant human AR was expressed in Sf9 cells via baculovirus vector pVL1393 as a FLAG-tagged fusion protein and purified on M2 agarose (57). His6-tagged p44 was expressed in bacteria via expression vector pET15d and purified by affinity (Ni-NTA agarose) and S-Sepharose chromatographic steps. The cDNA encoding the amino acid residues 1 to 282 of human AR was subcloned into vector pET15d and expressed in bacteria. The His6-tagged AR(1-282) protein was purified through Ni-NTA agarose column. Ten milligrams of the purified recombinant His6-tagged p44 and AR(1-282) proteins were sent to Convance Inc. (Denver, PA) for polyclonal antibody production in rabbits. The antisera were purified through the p44- and AR(1-282)-agarose columns, respectively.
**Transient transfection.** The AR, ER, GR, progesterone receptor (PR), TR and p44 expression vectors for transfection assays were constructed by inserting their corresponding cDNA sequences into pcDNA3.1. The luciferase reporters contain the androgen-, estrogen- or thyroid hormone-response elements ahead of the E4 basal promoter and the luciferase gene, respectively. PC3 cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum. Transfections were performed using Lipofectamine Reagent (Invitrogen, Carlsbad, CA). Briefly, $10^5$ cells were plated onto each well of 24-well plates approximately 24 h before transfection. After being washed with phosphate-buffered saline (PBS), cells in each well were transfected with 30 ng of an expression vector (AR, ER, GR, PR, or TR), 100 ng of the reporter plasmids, 2.5 ng of the pR-LUC internal control plasmid, and the indicated amount of the p44 expression vector. The total amount of DNA was adjusted to 300 ng with pcDNA3.1. Transfections were conducted in phenol-free RPMI 1640 medium; 2 h later, the medium was changed to either phenol-free RPMI 1640 plus 10% charcoal-treated feral bovine serum or regular medium containing 10 nM R1881, 10 nM dexamethasone, 10 nM progesterone, 1 µM β-estradiol, or 10 nM T3. Cells were cultured for another 48 h and harvested for the dual luciferase assay (Promega, Madison, WI).

**Protein-protein interaction assay.** One microgram of recombinant glutathione-S-transferase (GST) and GST-fusion proteins (GST-p44, GST-NTD, GST-DBD and GST-LBD) were expressed in bacteria and immobilized on 20 µl of glutathione Sepharose beads. The beads were incubated with 5 µl of rabbit reticulocyte lysate containing $^{35}$S-labeled AR, NKX3.1, or PRMT5 in a final volume of 200 µl containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 150 or 300 mM KCl, and 0.1% NP40. The beads were washed five times...
(1 ml each) with the incubation buffer, boiled in 20 μl of the SDS gel sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

**Chromatin Immunoprecipitation.** LNCaP cells were grown in phenol red-free RPMI 1640 supplemented with 10% charcoal/dextran-stripped fetal bovine serum for 2 days and then treated with 1 nM R1881 for 16 h. Cells were treated with ethanol were used as the control. Chromatin immunoprecipitation was performed as described (39) with the following modification. Cells were cross-linked with 1% formaldehyde at room temperature for 10 min and the cross-linking reaction was stopped by addition of glycine to 0.125 M. The cross-linked chromatin was sonicated with a Branso Sonifier 450 microtip at power setting 6 for five 30s bursts separated by cooling on ice. This treatment produced DNA fragments of average size of 700 bp. For immunoprecipitation, 2 μg of antigen-purified anti-AR or anti-p44 antibody was mixed with 300 μg of the purified cross-linked chromatin and incubated overnight at 4 °C. Immunocomplexes were washed five times (10 min each) in 1 ml of the buffer containing 1% Triton X100, 0.1% Na-deoxycholate, 0.05% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonylfluoride (PMSF); once in a solution containing 0.25 M LiCl, 0.5% NP40, 0.5% Na-deoxycholate, 1 mM EDTA, and 10 mM Tri-HCl, pH 8.0; and twice in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. After reversal and recovery of the immunoprecipitated chromatin DNA, the final DNA pellets were dissolved with 50 μl H2O. Immunopurified DNA (5 μl) was used for a PCR reaction (30 cycles, annealing at 50°C), with primers as follows. For prostate-specific antigen (PSA), the forward primer sequence was TCTGCCTTTGTCCGCTAGAT and the reverse primer sequence was AACCTTCATTCCCCAGGACT, which will amplify a 212-bp product from -250 to -39 upstream of the PSA transcription start site. For β-actin, the forward primer sequence was TCCTCCTCTTCTCTAAATCTCG and the reverse primer sequence was AAGGCAA
CTTTCGGAACGG, which will amplify a 145-bp product from -118 to -974 of the β-actin gene (the A of the ATG translation start codon was arbitrarily given the number +1).

**Methylation of proteins.** A cDNA (IMAGE:3833019) encoding the full-length human PRMT5 was purchased from ATCC. The point mutant (R368A) was created by using QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. The mutation was confirmed by DNA sequencing analysis. The wild-type and mutant PRMT5 were expressed in bacteria via pET15d expression vector and purified through Ni^{2+}-NTA agarose. The methylation assay was performed as follows. 2 μg of the purified histones (27) was incubated with 0.8 μg of the purified recombinant wild-type or mutant PRMT5 and various factors as indicated in 25-μl of 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 20 mCi ^3H-AdoMet (Amersham Pharmacia Biotech) at 30 °C for 30 min. The reactions were stopped by the addition of 5 μl of 5x SDS-sample loading buffer, and samples were resolved by 15% SDS-PAGE. The gels were stained with Coomassie blue R250, destained, treated with intensify solution, and analyzed by autoradiography.

**In situ hybridization.** We used matched normal and cancerous prostate tissues derived from radical prostatectomies of patients with prostate cancer at New York University Medical Center in an institutional review board-approved protocol. The procedure for in situ hybridization was as described previously (28). Briefly, the sections were hydrated, postfixed in 4% paraformaldehyde, and treated with proteinase K followed by deacetylation. The prehybridization and hybridization treatments were performed at 68°C using 0.3 M NaCl and 50% formamide. 500-bp (cDNA sequences of p44 from 1 to 500) DNA fragments containing both T7 and T3 promoters were generated by PCR. Corresponding ^32P-labeled RNAs (sense and
antisense) were generated by in vitro transcription with T7 and T3 RNA polymerases, respectively, and hybridized to the tissue sections (4 μm). After washing, the slides were exposed to NTB-2 X-ray emulsion (Eastman-Kodak, Rochester, NY) for 2-3 weeks, and counterstained with hematoxylin-eosin. Image and statistical analyses were performed as described (28).
RESULTS

Immunopurification and functional analysis of FLAG-AR-associated factors. We have previously generated stable cell lines expressing FLAG(41)-epitope-tagged factors and used them to affinity purify corresponding parental complexes (53, 54, 57). To similarly identify androgen-dependent or androgen-independent AR-interacting factors, we generated a prostate cancer cell line (f:AR-LNCaP) that stably expresses a FLAG-tagged AR. Immunopurification of f:AR from nuclear extracts made from f:AR-LNCaP cells grown in the presence and absence of androgen (R1881) revealed androgen (R1881)-induced association of a 44-kDa f:AR-associated polypeptide (Fig. 1A, lane 3). The specific association of this polypeptide with f:AR is further shown by the failure of similar-sized polypeptides in extracts from control cells (not expressing f:AR) to bind to the affinity matrix (Fig. 1A, lane 4).

When assayed in the purified minimal transcription system containing TFIIA, TFIIB, TFIID, TFIIH, pol II, and PC4 with the synthetic ARE-containing template (57) (Fig. 1C), recombinant AR (rAR) alone elicited up to 8.3-fold activation (Fig. 1B, lanes 6-8). The f:AR complex from nuclear extract derived from f:AR-expressing LNCaP cells grown in the presence of R1881 [f:AR(+)] elicited up to 38-fold activation (Fig. 1B, lanes 2 and 3), and at an equimolar input, was 16-fold more active than rAR (Fig. 1B, lane 3 versus lane 7). In contrast, the f:AR complex from nuclear extract from f:AR-expressing LNCaP cells grown in the absence of R1881 [f:AR(-)] showed a level of activity only about 2-fold above that shown by rAR (Fig. 1B, lanes 4 and 5 versus lanes 6 and 7). The amounts (as indicated in Fig. 1B) of recombinant AR and f:AR in the f:AR-containing complexes were normalized by quantitative Western blot analysis with anti-AR polyclonal antibodies. These results indicate that polypeptides associated with AR specifically in the presence of androgen can upregulate AR
function; a likely candidate is the 44 kDa protein, although other minor polypeptides could also be responsible. When the cell line was further expanded, the 44-kDa polypeptide became less abundant, and an attempt to isolate a sufficient amount of the 44-kDa polypeptide for peptide sequence analysis failed.

**Purification, cloning, and characterization of FLAG-NKX3.1-interacting proteins.** We have similarly generated a prostate cancer cell line (f:NKX3.1-LNCaP) that stably expresses a FLAG-tagged NKX3.1. Immunoprecipitation of f:NKX3.1 from extracts isolated from f:NKX3.1-LNCaP cells revealed 44- and 170-kDa polypeptides that specifically associate with NKX3.1 in the cytoplasm (Fig. 2A, lane 5), but not with NKX3.1 in the nucleus (Fig. 2A, lane 3). The specific association of these polypeptides with f:NKX3.1 is further shown by the failure of similar-sized polypeptides in extracts from control cells (expressing f:SRC1) to bind to the affinity matrix (Fig. 2A, lane 4). Because f:NKX3.1 stained negatively with silver, Western blot analysis with the anti-FLAG antibody was employed to demonstrate the existence of f:NKX3.1 in immunoprecipitates derived from both nuclear (Fig. 2A, bottom panel, lane 3) and cytoplasm (Fig. 2A, bottom panel, lane 5) extracts. After accumulating larger amounts of f:NKX3.1-associated 44-kDa protein, we performed direct sequence analysis by mass spectrometric methods (37). On the basis of the peptide sequence (KETPPPLVPPAAR) obtained from mass spectrometric analysis, we obtained a cDNA encoding the 44 kD protein. The p44 cDNA encodes a protein containing 342 amino acid residues and 4 putative WD-40 repeats (residues 68-107, 114-153, 157-196, and 280-319). P44 is identical in sequence to the recently identified MEP50 component of the methylosome (18) and to the WD45 subunit of the SME complex (35). Northern blot analysis of multiple human tissues showed that p44 mRNA is highly expressed in the heart, skeletal muscle, spleen, testis, uterus, prostate, and thymus (Fig.
3). Western blot analysis with anti-p44 antibody revealed that the f:AR preparation immunopurified from the f:AR-LNCaP cell line (Fig. 1A, lane 3) contains the same 44-kDa polypeptide (Fig. 2B, lane 3).

**P44 specifically enhances AR-dependent transcription in vivo.** To investigate the effect of p44 on AR-dependent transcription in vivo; a luciferase reporter containing four tandem prostate-specific antigen (PSA) promoter AREs (-152 to -174) (14) upstream of the minimal adenovirus E4 promoter (-38 to +93) was cotransfected with expression vectors for AR and p44 into PC3 cells in the absence or presence of ligand (R1881). As shown in Fig. 4A, AR activated the reporter gene about 12-fold in the presence of ligand, and coexpressed p44 showed a strong (up to 3.3-fold) enhancement of this activity that is likely restricted in magnitude by contributions from endogenous p44 (data not shown). P44 did not influence reporter gene activity in the absence of cotransfected AR or ligand (R1881), indicating that the enhancing effect of p44 on AR-dependent gene expression was caused by an effect on the E4 promoter. To investigate the receptor specificity of p44, we examined the effects of p44 on transcription of reporters containing the same E4 promoter under the control of GR, progesterone receptor (PRb), estrogen receptor (ERα) and thyroid hormone receptor (TR). As shown in Fig. 4A, p44 also enhanced GR- and PR-driven gene expression and, in contrast, showed no effect on TR- or ER-mediated transcription. Hence, p44 shows some nuclear receptor-specific effects in vivo. P44 also enhanced AR-driven transcription from natural ARE-containing MMTV (15) and probasin (-244 to +12) (13) promoters but had no obvious effects on the promoter derived from the PSA enhancer (-4354 to -3858) (47) (Fig. 4B). These results suggest that p44 has promoter specificity.
P44 interacts directly with AR and NKX3.1. Consistent with the observed intracellular association of p44 with f:NKX3.1 (Fig. 2A, lane 5), p44 interacted directly and strongly with NKX3.1 in a salt-insensitive manner in a GST pull-down assay (Fig. 5A, lanes 10-14). Recombinant AR was also found to interact strongly (but in a salt-sensitive manner) with a GST-p44 fusion protein but not with GST alone (Fig. 5A, lanes 1-9), which was consistent with the intracellular association of f:AR with p44 (Fig. 1A, lane 3). The ligand-independent in vitro interaction of purified p44 with purified AR contrasts with the ligand-dependent intracellular association of p44 with AR. However, other AR-associated proteins (such as heat shock proteins) whose interactions are reversed by androgen, thus facilitating p44 interaction, may explain this dependency. Our identification of a common interacting protein (p44) prompted studies of the effect of NKX3.1 on AR function, which showed that overexpression of NKX3.1 represses AR-driven gene expression in vivo (Fig. 2C). One possible explanation is that NKX3.1 may sequester p44 in the cytoplasm, therefore inhibiting AR-driven gene expression. Overexpression of ectopic p44 was shown to completely overcome the NKX3.1-mediated repression, which supports this theory (Fig. 2C). Since p44 enhances AR activity either in the presence or absence of NKX3.1, the mechanism of enhancement by p44 may be completely independent of NKX3.1 action.

P44-containing complex enhances AR-driven transcription in vitro. To further explore the function and regulation of p44, we established a HeLa cell line that stably expresses FLAG-tagged p44 and used it to immunopurify p44-containing complexes. SDS-PAGE analysis revealed a large number of polypeptides (more abundant in the 50- to 60-kDa range and less abundant in the 70- to 100-kDa and 10- to 30-kDa ranges) (Fig. 5B, lane 5). The recombinant p44 expressed in bacteria (Fig. 5B, lane 2) inhibited AR-dependent transcription from the
synthetic ARE-E4 promoter (Fig. 5C, lane 3 versus lane 2). This repression contrasted with the activation observed in vivo (transient transfection) (Fig. 4). One possible reason for this is that p44 activation of AR-driven gene expression requires additional factors. In the absence of these factors in the in vitro reconstituted transcription system, p44 might sequester some transcription factors (such as AR) through direct interactions and thus repress transcription. This hypothesis is supported by our finding that the p44-containing complex purified from the FLAG-tagged stable cell line enhanced AR-driven transcription from the same promoter (Fig. 5C, lane 5). The p44-containing complex did not affect GAL4-VP16-driven transcription in the same in vitro transcription system (data not shown).

The occupancy of specific DNA sites by specific DNA binding proteins (e.g., AR) and associated proteins can be established by the ChIP assay (38). This assay is a direct and powerful method of assessing in vivo protein-DNA interactions. As shown in Fig. 5D, AR binds to the PSA promoter region in the presence of androgen (R1881) (middle panel, lane 1). As negative controls, the products amplified by PCR at the same time from the β-actin promoter were not changed in response to the addition of androgen (middle panel, lanes 3 and 4). This observation is consistent with the fact that the PSA promoter is directly targeted by AR (58). We have performed this assay for over 10 times and consistently observed the androgen-dependent recruitment of AR to the PSA proximal promoter in LNCaP cells. Cofactors can also be cross-linked by formaldehyde treatment to chromatin through their interactions with DNA-binding factors in living cells. Therefore, the ChIP assay is also a direct way to determine cofactor occupancy on AR-target genes. Fig. 5D shows the androgen-dependent recruitment of p44 onto the PSA promoter (bottom panel, lane 1 versus lane 2). The ChIP assay with anti-p44 antibody was independently performed twice and the results were consistent. We found that when the
increased amounts (2-fold) of DNA were used in our standard PCR reactions (30 cycles) we still observed the androgen-dependent recruitment of p44 to the PSA promoter although the background was slightly higher. However, less amplification cycles (27 cycles) in the PCR reaction would give the better results when the increased amounts of DNA were used. These results suggest that p44 functions on the AR-target gene in vivo.

Others have been reported that MEP50 and WD45 form complexes with PRMT5 and pICln in the methylosome and SMN complexes, respectively (18, 35). To further establish whether these latter two proteins are present in our p44-containing complex, Western blot analysis with anti-PRMT5 and anti-pICln antibodies was performed. As shown in Fig. 5B, both PRMT5 and pICln proteins were present in the p44-containing complex (lane 7). To determine whether PRMT5 and pICln are involved in AR-driven gene expression, we subcloned cDNAs encoding PRMT5 and pICln (IMAGE:3836445) (ATCC) into the expression vector pcDNA3.1. To investigate the effect of PRMT5 and pICln on AR-dependent transcription in vivo, an ARE-containing luciferase reporter was cotransfected with expression vectors for AR, PRMT5, pICln, or different combinations into prostate cancer PC3 cells in the presence of ligand (R1881). As shown in Fig. 6, AR activated the reporter gene about 12-fold, and co-expressed PRMT5 showed a strong (up to 2.5-fold) enhancement of this activity. PRMT5 did not influence reporter gene activity in the absence of cotransfected AR or ligand (R1881) (data not shown), indicating that the enhancing effect of PRMT5 on AR-dependent gene expression was caused by an effect on the E4 promoter. To investigate the effect of PRMT5 plus p44 in the same assay, we cotransfected PC3 cells with limited amounts (50 ng) of PRMT5 and p44 alone or in combination. Fig. 6 shows that 50 ng of PRMT5 or p44 had little effect on AR-dependent transcription. However, the same amounts of combined PRMT5 plus p44 resulted in strong (3-
fold) activation, indicating that PRMT5 and p44 function synergistically. In contrast, pICln alone or in combination with p44, PRMT5, or both had no significant effect on AR-dependent transcription (Fig. 6). Western blot analysis indicated that f:AR complex (Fig. 1A, lane 3) also contains PRMT5 and pICln (data not shown).

The methyltransferase activity of PRMT5 is not required for the enhanced transactivation of AR. We further studied the interactions among AR, p44, and PRMT5. In vitro produced and $^{35}$S-labeled PRMT5 was incubated with immobilized GST, GST-p44, GST-NTD, GST-DBD, and GST-LBD. After wash, bound proteins were resolved by SDS-PAGE and visualized by autoradiography. P44 directly interacted with PRMT5 (Fig. 7A, lane 3). In contrast, PRMT5 did not bind any domain (NTD, DBD, or LBD) of AR (Fig. 7A, lanes 7-9). These results indicate that PRMT5 is recruited to AR-target genes through its direct interaction with p44. The conserved arginine residue (R368) is essential for the methyltransferase activity of PRMT5 (40). The wild-type (Fig. 7B, lanes 2 and 3) and R368A mutant (lanes 4 and 5) PRMT5 were expressed in and purified from bacteria. Incubations of histones purified from HeLa cells with $^3$H-AdoMet plus recombinant PRMT5 resulted in transfer of the radioactive methyl groups to the histone H4 (Fig. 7C, top panel, lane 2). In contrast to the previous observations (40), we did not detect the methylation of the histone H2A by PRMT5. This discrepancy might be due to the different preparations of histones used for the methylation assay. The preparation of individual histones was used in the previous study but the purified natural histones (containing the octomer of 2H2A, 2H2B, 2H3, and 2H4) were used in our study. The mutation of R368A on PRMT5 dramatically reduced the methyltransferase activity in vitro (Fig. 7C, top panel, lane 3 versus lane 2). However, this mutation did not significantly decrease the PRMT5-mediated transactivation of AR in vivo (Fig. 7D), indicating that the
methyltransferase activity of PRMT5 is not required. Western blot analysis with anti-PRMT5 antibody revealed that the protein levels of the wild-type and mutant PRMT5 in the transfected PC3 cells are same (data not shown). However, the same mutation on PRMT5 impaired its activity as transcriptional corepressor on the cyclin E1 promoter (16).

**Overexpression of p44 correlates with prostate tumorigenesis.** In order to investigate the possibility that p44 might be important for modulating AR function in prostate cancer, we investigated the expression of p44 at the mRNA level using quantitative in situ hybridization methods in 43 primary prostate cancers with different degrees of differentiation. Expression of p44 was detected in only prostate epithelial cells (EC) and was significantly up-regulated in 36% of well-differentiated prostate tumors (Fig. 8, a, b, g, h), in more than 80% of moderately differentiated prostate tumors (c, d, i, j), and in more than 60% of poorly differentiated prostate tumors (e, f, k, i). The average increase-fold is 5.1. These results indicate that changes in p44 expression (and possibly in associated proteins) might play an important role in effecting the deregulation of normal AR and NKX3.1 functions in prostate tumorigenesis.
DISCUSSION

In this study, we described the isolation of p44 as a new AR- and NKX3.1-interacting protein both in vitro and in vivo. Transient transfection assays demonstrated that p44 increased AR transcriptional activity in an androgen-dependent manner. P44 forms a multiprotein complex that enhanced AR-dependent transcription in a cell free transcriptional system.

A novel cofactor complex functions as an AR coactivator. Increasing numbers of cofactors have been indicated in the function of AR (22, 24). They are identified through their physical interactions with AR and enhanced or repressed AR-mediated transcription in vivo. Our attempt to isolate the AR-associated proteins from stable cell lines resulted in the identification of p44. P44 and p44-containing complex enhanced AR-dependent transcription in vivo and in vitro, respectively. The protein sequence of p44 is identical to that of a component (MEP50) of the methylosome (18) and a subunit (WD45) of the SMN complex (34). The methylosome complex contains PRMT5/JBP1, pICln, and Sm proteins and mediates the assembly of spliceosomal snRNP (17). MEP50 is important for methylosome activity and binds to PRMT5/JBP1 and to a subset of Sm proteins (18). SMN is part of a complex contains the Sm proteins and PRMT5 and necessary and sufficient for assembly of UsnRNA (35, 48). The methylosome and SMN complexes were isolated from the cytoplasm of HeLa cells, and the p44-containing complex was purified from the HeLa nuclear extract (17, 35). Thus, p44 may form distinct complexes with different proteins in the cytoplasm and in the nucleus for different roles (transcription versus splicing/translocation). The apparent size of MEP50 (above that of the 45-kDa bovine serum albumin) revealed by SDS-PAGE (17, 18) is larger than that of p44 and WD45 (below that of the 45-kDa bovine serum albumin), indicating that posttranslational modifications may exist in MEP50.
PRMT5 is present within the p44-containing complex. Two types of PRMT activities have been identified in mammalian cells (59). PRMT1, PRMT2, and PRMT4/CARM1 have been found to participate in nuclear receptor transcriptional activation (43, 49, 51). The methylation of histones H3 and H4 by PRMT1 and PRMT4/CARM1 correlates with transcriptional activation, suggesting that they act by modifying chromatin structure. More recently, PRMT5 was identified as a corepressor of cyclin E1 transcription (16). Forced expression of PRMT5 negatively affected cyclin E1 promoter activity, which required the methyltransferase activity of PRMT5 (16). In contrast, our results demonstrate that PRMT5 is a positive AR cofactor that functions in a methyltransferase activity-independent manner in transient transfection assay. Since the reporter gene in the transient transfection is likely not well packaged into chromatin we cannot rule out the involvement of the methyltransferase activity of PRMT5 in AR function on the genes integrated in chromatin. Similarly, PRMT2 was identified as a methyltransferase based on the protein sequence and functioned as a positive cofactor for estrogen receptor $\alpha$, but so far its methyltransferase activity has not been identified with substrates including histones and estrogen receptor $\alpha$ (43). The enhancement of AR-dependent transcription by PRMT5 might result from activation domains existing within PRMT5 protein or from the structural role of PRMT5 required for assembling the p44-containing cofactor complex. The former possibility is not supported by that fact that no activation was observed when PRMT5 was tethered to DNA through the DNA-binding domain of GAL4 (data not shown).

P44 is overexpressed in prostate cancer. The observation that certain cofactors are abnormally expressed in some prostate cancers indicates the importance of nuclear receptor cofactors in transcriptional control of AR function and also points to their possible role in
neoplastic conversion (28). Overexpression of p44 in prostate cancer tissues indicates that it may play an important role in prostate tumorigenesis and there is well documented evidence that abnormal NKX3.1 expression is involved in prostate tumorigenesis. Our funding that p44 interacts with both NKX3.1 and AR suggests that it might play a role in coregulating these two pathways.

In summary, our results point to a novel cofactor complex in the regulation of AR-dependent transcription. AR is an important regulatory factor in the development, differentiation, and maintenance of male reproductive functions, as well as in the regulation of other sexually dimorphic processes ranging from the development of neural tissues to the modulation of immune function. Thus, the p44-containing complex may play a pivotal role in these biological processes by modulating the transcriptional activity of AR.
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REFERENCES


FIGURE LEGEND

FIG. 1. Effects of affinity-purified f:AR-cofactor complexes on transcription in a system reconstituted with purified factors. (A) SDS-PAGE analysis of f:AR-cofactor complexes. Lanes 1 and 3 show AR-containing complexes immunopurified from nuclear extracts made from a stably transfected, FLAG-tagged, AR-expressing LNCaP cell line grown in the presence (lane 3) or absence (lane 1) of the synthetic androgen R1881 (10 nM). The gel was stained with silver. Bands corresponding to FLAG-tagged AR (f:AR) and to the polypeptide specifically associated with AR are indicated by the arrows at left. (B) AR- and AR-cofactor-dependent transcription. A synthetic template containing four ARE elements (panel C) was transcribed in the system reconstituted with purified factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, Pol II, and PC4) with additions of the rAR and f:AR-cofactor complexes described in panel A. The specifically initiated transcript is indicated by an arrow and was monitored by primer extension. The fold-activation relative to levels of transcription in the absence of rAR or f:AR complexes (lane 1) is indicated at the bottom of the panel. (C) Diagram of the synthetic ARE-containing template. The template (pARE-E4) contains four tandem copies of the ARE from the prostate-specific antigen promoter positioned upstream of the adenovirus E4 promoter.

FIG. 2. P44 associates with NKX3.1 in the cytoplasm. (A) SDS-PAGE analysis of purified f:NKX3.1-containing complex. Immunoprecipitation was performed with nuclear extracts (lane 3) and cytoplasmic extracts (lane 5) made from a stably transfected, FLAG-tagged, NKX3.1-expressing cell line. Bands corresponding to FLAG-tagged NKX3.1 and polypeptides specifically associated with NKX3.1 (p44 and p170) are indicated by the arrows at right. The specific association of these polypeptides with f:NKX3.1 is further shown by the failure of similarly sized polypeptides in extracts from control cells (expressing f:SRC1) to bind to the
affinity matrix (lanes 2 and 4). Lane 1 has standard molecular weight markers (Bio-Rad). The bottom panel is a western blot analysis of the same samples with anti-FLAG monoclonal antibody. The FLAG-tagged NKX3.1 is indicated by the arrow at right. (B) Western blot analysis of the f:AR-complexes with anti-p44 antibody. The band corresponding to p44 is indicated by the arrow at right. Lane 1 contains 5 μl of nuclear extract made from LNCaP cells. (C) NKX3.1 partially represses AR-dependent gene expression, and the over-expression of p44 relieves this repression. PC3 cells were transfected with 100 ng of 4 x ARE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, 60 ng of pcDNA NKX3.1, and 100 ng of p44, as indicated. Cells were grown in the presence of 100 nM R1881 for 48 h after transfection, and then harvested for luciferase activity assays.

FIG. 3. Northern blot analysis of p44 expression. The membrane in the top panel was probed with $^{32}$P-labeled p44 cDNA, and the bottom panel shows the same membrane probed with $^{32}$P-labeled β-Actin cDNA.

FIG. 4. P44 specifically enhances AR-mediated transcription in vivo. (A) P44 enhanced AR, GR, and PR-mediated transcription. PC3 cells were transfected with 100 ng of 4 x ARE-, 3 x ERE-, or 2 x TRE-E4-luc reporter plasmid; 30 ng of pcDNA-AR, -GR, -PR, -ER, or -TR, and indicated amounts of pcDNA-p44 expression plasmid. Cells were grown in the absence or presence of 10 nM R1881, 10 nM dexamethasone, 10 nM progesterone, 1 μM estradiol, or 10 nM T3 for 48 h after transfection and then harvested for luciferase activity assays. (B) P44 selectively affected AR-mediated luciferase gene expression from different promoters. PC3 cells were transfected with 100 ng of MMTV-, probasin-, or PSA(I)-luc reporter plasmid, 30 ng of pcDNA-AR, and 150 ng of pcDNA-p44 expression plasmid, as indicated. Cells were grown in
the presence of 10 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays.

FIG. 5. P44-containing complex enhances AR-driven transcription. (A) p44 interacts directly with AR and NKX3.1. GST-p44 fusion protein expressed in bacteria was immobilized on glutathione agarose beads. Beads were incubated with $^{35}$S-labeled AR (lanes 1-9) or NKX3.1 (lanes 10-14) in BC150-0.1% NP40 (lanes 2, 3, 6, 7, 11, and 12) or BC300-0.1% NP40 (lanes 4, 5, 8, 9, 13, and 14) in the absence (lanes 2-5, 10-14) or presence (left panel, lanes 6-9) of 50 nM R1881 for 2 h at 4 °C. After washing with the incubation buffer, the beads were boiled with SDS sample buffer and subjected to SDS-PAGE followed by autoradiography. (B) SDS-PAGE analysis of purified p44 and p44-containing complexes. Lane 2 shows recombinant p44 expressed in bacteria and purified on an Ni-NTA agarose affinity column; lanes 4 and 5 show p44-containing complexes immunopurified from nuclear extracts made from a stably transfected, FLAG-tagged, p44-expressing HeLa cell line and immunoprecipitate from extracts made from control cells (not expressing f:p44), respectively. The gels were stained with Coomassie blue R250. Bands corresponding to p44 or FLAG-tagged p44 (f:p44) are indicated by the arrows at right. Polypeptides specifically associated with p44 are indicated by short lines at the right (lane 6). Lanes 1 and 3 are standard molecular weight markers (Bio-Rad). Lanes 7 and 8 show a Western blot analysis of the immunoprecipitate isolated from p44-expression cells (lane 7) and control cells (lane 8) using anti-PRMT5 and anti-pICln antibodies. (C) The p44-containing complex enhances AR-dependent transcription. A synthetic template pARE-E4 was transcribed in the system reconstituted with purified factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, Pol II, and PC4) with additions of rAR, p44, and the f:p44-containing complex described in panel A. The specifically initiated transcript is indicated by an arrow and was monitored by primer extension.
(D) P44 was recruited on the PSA promoter in the presence of the androgen. LNCaP cells were grown in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 1 nM R1881. ChIP assay was performed with antigen-purified anti-AR (middle panel) or anti-p44 (bottom panel) antibodies. The purified protein-DNA cross-links were reversed and the resulting DNA was amplified by a PCR reaction with two specific primers derived from promoter regions of PSA (lanes 1 and 2) or β-actin (lanes 3 and 4). The same set of PCR reactions was performed with chromatin DNA (Input) used for ChIP assay (top panel).

FIG. 6. PRMT5 synergizes with p44 to enhance AR-driven gene expression. PC3 cells were transfected with 100 ng of ARE-E4-luc reporter plasmid, 30 ng of pcDNA-AR, and indicated amounts of pcDNA-p44, -PRMT5, or -pICln expression plasmid. Cells were grown in the absence or presence of 10 nM R1881 for 48 h after transfection and then harvested for dual luciferase activity assay.

FIG. 7. (A) p44 physically interacted with PRMT5. The $^{35}$S-labeled PRMT5 incubated with the indicated GST-fusion proteins. After wash, bound proteins were resolved by 10% SDS-PAGE and visualized by autoradiography. Lanes 1 and 5 are 10% of the labeled PRMT5 used in binding reactions. (B) SDS-PAGE analysis of recombinant wild-type (lanes 2 and 3) and R368A mutant (lanes 4 and 5) PRMT5 expressed in bacteria and purified on an Ni-NTA agarose affinity column. The gel was stained with Coomassie blue R250. Lane 1, the standard molecular weight markers (Bio-Rad). (C) in vitro methyltransferase assay. The methyltransferase assay was performed as described under “Materials and Methods”. Top panel, autoradiography of the gel; bottom panel, Coomassie Blue staining of the same gel. Individual histones are indicated on the left. (D) The methyltransferase activity is not required for PRMT5 function on AR-driven gene expression. PC3 cells were transfected with 100 ng of 4 x ARE-E4-luc reporter plasmid, 30 ng
of pcDNA-AR, and 150 ng of pcDNA-PRMT5 or pcDNA-PRMT5(R368A) expression plasmid as indicated. Cells were grown in the presence of 10 nM R1881 for 48 h after transfection and then harvested for luciferase activity assays.

FIG. 8. Enhanced expression of p44 in prostate tumor tissues. (A) Four-micron-thick frozen sections of prostate tissues were prepared and kept frozen until used. The frozen tissue sections were fixed in 4% paraformaldehyde for 30 min, dehydrated with ethanol and hybridized with antisense p44 RNA probes in vitro labeled with α-32P-UTP. The slides were first washed with 2x SSC at room temperature, then twice with 0.2X SSC at 45 °C for 20 min. The slides were exposed and evaluated using Nikon microscope with a digital camera interfaced to a computer. (B) Quantitative data for the in situ hybridization analysis.
Figure 2
Figure 3
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Figure 4A
Figure 4B
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Figure 6
A  

benign tissue

well differentiated

tumor tissue

moderately differentiated

poorly differentiated

B  

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