Identification of Putative Androgen Receptor Interaction Protein Modules

CYTOSKELETON AND ENDOSONES MODULATE ANDROGEN RECEPTOR SIGNALING IN PROSTATE CANCER CELLS

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We have developed a novel androgen receptor (AR) expression system in the 293 human embryonic kidney cell line that recapitulates AR biochemical activity as a steroid hormone receptor in prostate cancer cells. We used this system to identify putative AR-binding proteins in the cytosolic and nuclear compartments of mammalian cells using a large scale co-immunoprecipitation strategy coupled to quantitative mass spectrometry. For example, the heat shock 70 and 90 chaperones, which are known regulators of steroid hormone receptor, were identified as AR-binding proteins. AR purification enriched for proteins involved in RNA processing, protein transport, and cytoskeletal organization, suggesting a functional link between AR and these protein modules in mammalian cells. For example, AR purification in the nuclear compartment led to the specific enrichment of α-actinin-4, clathrin heavy chain, and serine-threonine protein kinase C δ. Short interfering RNA knockdown studies and co-transcriptional reporter assays revealed that clathrin heavy chain possessed co-activator activity during AR-mediated transcription, whereas α-actinin-4 and protein kinase C δ displayed both co-activator and co-repressor activity during AR-mediated transcription that was dependent upon their relative expression levels. Lastly immunohistochemical staining of prostate tissue showed that α-actinin-4 levels decreased in the nucleus of high grade cancerous prostate samples, suggesting its possible deregulation in advanced prostate cancers as previously observed in late stage metastatic breast cancers. Taken together, these findings suggest AR binds to specific protein modules in mammalian cells and that these protein modules may provide a molecular framework for interrogating AR function in normal and cancerous prostate epithelial cells. Molecular & Cellular Proteomics 6: 252–271, 2007.

Androgens, acting through the steroid hormone androgen receptor (AR), play a critical role in the development and maintenance of the normal prostate gland (1). Androgens also influence the initiation and progression of prostate cancer (PCa) (2) as AR expression and activity promote the growth of late stage PCa (3). AR is a member of the nuclear family of steroid hormone receptors (SHRs) (1), a family of transcription factors that consist of a ligand-binding domain, a conserved DNA-binding domain, and a transcriptional activation domain (1). In the absence of androgens, AR typically resides in the cytosol as a protein complex bound by chaperones (4). Upon binding androgens, AR dissociates from chaperones and translocates to the nucleus where it binds to androgen-response elements located in the promoters of targeted genes (4–6). AR binding to DNA facilitates the recruitment of general transcriptional machinery and ancillary factors that results in the activation or repression of specific genes in targeted cells and tissues (7–10).

Co-regulators are functionally classified as co-activators (e.g. SRC-1, SRC-2, and SRC-3) or co-repressors (e.g. Nuclear Receptor co-Repressor Silencing Mediator of Retinoic Acid and thyroid hormone receptor) based upon their ability to positively or negatively regulate SHR-mediated transcription (11). Perturbations in the expression of co-regulators repre...

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Received, May 8, 2006, and in revised form, August 31, 2006.

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This paper is available on line at http://www.mcponline.org
sents a plausible mechanism of prostate tumorigenesis in men (12). Many proteins can function as co-regulators of AR-mediated transcription as they modulate AR-mediated transcription through multiple mechanisms that include the recruitment of chromatin-remodeling complexes, influence AR ligand specificity and stability, or regulate AR localization and trafficking in mammalian cells (11). Besides the well characterized AR genomic activity as a transcriptional regulator, AR also possesses non-genomic activity (e.g. oocyte maturation) (13). Androgens elicit biological responses in mammalian cells on the time scale of seconds and minutes that purportedly occurs through a membrane-associated AR (14, 15). Proteins that bind AR are best known as co-regulators of AR-mediated transcription, but it would be reasonable to suspect this class of molecules will also influence AR non-genomic activity in mammalian cells (14).

All available data suggest that no single AR-binding protein will completely define AR multiple functions in controlling cellular growth and differentiation in normal and neoplastic prostate epithelial cells (1). Alternatively, AR pleiotropic activities will probably be mediated through its binding to specific functional protein complexes to carry out its broad biological functions in mammalian cells (16). Historically, yeast two-hybrid (Y2H) screens and directed in vitro protein binding assays have been used extensively to detect novel AR-binding proteins. For example, many of these studies routinely use specific AR domains (e.g. ligand-binding and DNA-binding domains) as targets to detect novel interacting proteins (18). Unfortunately this experimental design lacks the power to detect protein complexes that associate with AR as an intact molecule. Therefore, to broaden our knowledge of AR-binding proteins in mammalian cells, a novel AR expression system was developed to systematically identify AR-binding proteins in the cytosolic and nuclear compartments of mammalian cells in an unbiased manner. An isotopic protein labeling strategy using the ICAT method was used to identify putative AR-binding proteins by their enriched ICAT ratios relative to nonspecific protein components using MS/MS after AR purification. Interestingly AR purification specifically enriched for proteins involved in RNA processing, protein transport, and cytoskeletal processes. Several putative AR-binding proteins were characterized for further study, including clathrin heavy chain, α-actinin-4, and protein kinase C δ (PKC δ). siRNA knockdown experiments in PCa cells and co-reporter assays in mammalian cells revealed that clathrin heavy chain, α-actinin-4, and PKC δ are co-regulators of AR-mediated transcription. Furthermore, immunohistochemical staining of α-actinin-4 expression in human prostate tissue showed it was localized exclusively to the cytoplasm of late stage PCa, suggesting that α-actinin-4 localization may serve as a biomarker of advanced staged PCa. These findings provide a molecular framework for the characterization of AR-binding protein modules and they also provide a platform for developing new hypotheses about the AR role in the regulation of these molecular pathways in normal and cancerous prostate epithelial tissue.

**MATERIALS AND METHODS**

**Reagents**

The AR agonist R1881 (methytrienolone) was purchased from PerkinElmer Life Sciences. Double-stranded siRNAs were purchased from Dharmacon Research (Lafayette, CO); Oligofectamine reagent, 4–12% SDS-PAGE gels, and TEV protease were purchased from Invitrogen. Prestained Precision Plus protein standards were purchased from Bio-Rad. Corresponding antibodies used for these analyses included monoclonal AR antibody (AR441) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); rabbit polyclonal prostate-specific antigen (PSA) from DakoCytomation (Carpinteria, CA); monoclonal clathrin heavy chain (clone 23), monoclonal PKC δ (clone 14), β-spectrin II (clone 42), and nucleoporin p62 (clone 53) from BD Transduction Laboratories; rabbit polyclonal α-actinin-4 from Axxora (San Diego, CA); and monoclonal protein A (SPA-27) from Sigma. The drug geldanamycin A was purchased from Sigma. The BCA protein assay and NE-PER Nuclear and Cytoplasmic Extraction Reagents kits were purchased from Pierce. Full-length human IMAGE cDNA clones (PKC δ IMAGE clone I.D. 5539909, α-actinin-4 IMAGE clone I.D. 3842046, and clathrin heavy chain IMAGE clone I.D. 6187185) were purchased from American Tissue Type Culture Collection (ATCC) (Manassas, VA). The Dual-Luciferase reporter assay was purchased from Promega (Madison, WI). The anti-chitin-binding domain serum (Promega) and clathrin heavy chain IMAGE clone I.D. 3842046, and clathrin heavy chain IMAGE clone I.D. 6187185) were purchased from Chemicon (Temecula, CA). 4',6-Diamidino-2-phenyldole dihydrochloride (DAPI), Texas Red phalloidin, and the Alexa Fluor 488 goat anti-mouse secondary conjugate were purchased from Molecular Probes (Eugene, OR).

**Cell Culture and Cell Lines**

LNCaP cells were obtained from ATCC and maintained in normal growth medium containing phenol red-deficient RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) or 10% charcoal/dextran-treated FBS (Hyclone Laboratories Inc., Logan, UT). 293HEK cell lines were grown in phenol red-deficient, high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% FBS or 10% charcoal/dextran-treated FBS (medium D) supplemented with G418 sulfate at 1 mg/ml. All cultures were supplemented with penicillin/streptomycin/glutamine and maintained in a 37 °C incubator in 5% CO₂.

For the generation of NTAP, NTAP-AR, and CTAP-AR 293HEK cell lines, the pSG5-AR mammalian expression vector was used as a template to PCR subclone AR into the pcDNA3-NTAP and pcDNA3-CTAP mammalian expression vectors, respectively (19). AR was PCR-amplified using the Advantage GC-2 polymerase (Clontech) and cloned in-frame into the 5’ BamHI and 3’ NotI restriction sites of pcDNA3-NTAP and pcDNA3-CTAP vectors as N-terminal and C-terminal tandem affinity purification (TAP) fusion proteins accordingly. The oligonucleotide primers (Integrated DNA Technologies) used for cloning NTAP-AR consisted of a 5’ BamHI primer, GATGGGATCCATTGGAAGTTGCAGTTAGGGGAAAGGCTTAC, and a 3’ NotI primer, GATGCGGCGCCGCTCACTGGGTTGGAATAATAGATGGGCTTGCCTTAT.
CCCGAGAAG. The oligonucleotide primers used for cloning CTAP-AR consisted of the same exact 5′ BamH1 primer listed above and the following 3′ NotI primer: GATCCCAGCGGCCATCTGGGTGTTGCTTAAGATGGAATTCTTTCCAGAAG. Individual pcDNA3-NTAP-AR and pcDNA3-CTAP-AR cDNA clones were transiently transfected into 293HEK cells and probed with protein A (Sigma, SPA-27) and anti-chitin-binding domain serum (New England Biolabs) antibodies to verify NTAP-AR and CTAP-AR expression. Functional pcDNA3-NTAP-AR and pcDNA3-CTAP-AR mammalian expression vectors were DNA sequence-verified and used to generate stable NTAP, NTAP-AR, and CTAP-AR 293HEK cell clones by selection in G418, NTAP-, NTAP-AR-, and CTAP-AR-expressing cell clones were identified by Western blot screening using a mixture of antibodies against protein A and AR.

**NTAP and NTAP-AR Purification**

NTAP and NTAP-AR cells were grown in 10 separate 500-mm tissue culture plates (10 dishes) containing phenol red-deficient RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. Semi-confluent dishes were washed three times with PBS and grown for 48 h in androgen-depleted growth medium (AD) (10% charcoal-stripped bovine serum) to reduce androgen levels to the detection limit of our assay (200 fmol/L). Following washing, 20 bed volumes of buffer A (20 mM HEPES, 25% glycerol, 100 mM KCl, 2 mM EDTA, 5 mM ATP, 100 mM R1881, 5 mM DTT) followed by the addition of TEV protease (1000 units) (Invitrogen) and incubation at 30 °C for 8 h. 3 bed volumes of buffer A were added to each column, and the eluate was collected after a 5-min centrifugation at 500 rpm.

**Cytosolic TAP Protein Purification**—250 mg of NTAP and NTAP-AR cytosolic protein extracts were purified in parallel at 4 °C over a column packed with IgG-Sepharose 6 Fast Flow (GE Healthcare) beads (3-ml bed volume). The columns were washed with 20 bed volumes of buffer A (20 mM HEPES, 25% glycerol, 100 mM KCl, 2 mM EDTA, 5 mM ATP, 100 mM R1881, 5 mM DTT) followed by the addition of TEV protease (1000 units) (Invitrogen) and incubation at 30 °C for 8 h. 3 bed volumes of buffer A were added to each column, and the eluate was collected after a 5-min centrifugation at 500 rpm.

**Nuclear TAP Protein Purification**—250 mg of NTAP and NTAP-AR nuclear protein extracts were purified in parallel using the same exact binding and wash conditions used for the cytosolic TAP protein purification described above.

**ICAT Labeling and MS Analysis**

Equal amounts (200 μg/sample) of NTAP and NTAP-AR TEV-eluted cytosolic protein extracts and equal amounts (150 μg/sample) of NTAP and NTAP-AR TEV-eluted nuclear protein extracts were subjected to the ICAT method, respectively (20). NTAP and NTAP-AR protein extracts were labeled with the light (d5) and heavy (d6) ICAT reagent labels, respectively. Samples were digested with trypsin and subjected to strong cation exchange (400 × 2.1-mm column) chromatography and avidin purification as described previously (20). Approximately 30 ICAT-labeled peptide fractions were analyzed by microcapillary LC-ESI-MS/MS (20).

**SEQUEST, INTERACT, and XPRESS Scoring Criterion**

Uninterpreted MS/MS spectra contained in RAW files were acquired on an LCQ classic ion trap mass spectrometer and searched using the SEQUEST database search software (21) against a database of human sequences generated as a subset of a non-redundant amino acid database downloaded from the National Cancer Institute’s Advanced Biomedical Computing Center. Tandem mass spectra were analyzed using the SEQUEST database search criteria that included a static modification of cysteine residues of 503 Da (mass of cysteine plus light ICAT reagent) and a variable modification of 8 Da for cysteines (for the heavy ICAT reagent). Searches were performed with no enzyme constraint on the peptides analyzed from the sequence database, and the identified peptides were processed and analyzed through the mass spectrometry Transproteomic Pipeline. The Transproteomic Pipeline software includes a peptide probability score program called PeptideProphet that aids in the assignment of peptide MS spectrum and the ProteinProphet program that assigns and groups peptides to a unique protein or a protein family if the peptide is shared among several isoforms (22, 23). Lastly all peptide quantifications were analyzed using XPRESS (24). A ProteinProphet probability score of 0.95 was used for all accepted ICAT-labeled protein identifications. All quantified peptides were manually inspected and verified for authenticity.

**Immunoblotting, Immunofluorescence, and Silver Staining**

**Immunoblotting**—The exact experimental conditions used for generating protein lysates in LNCaP, NTAP, and NTAP-AR are detailed in the corresponding figure legends. In brief, cells were washed once with PBS and solubilized in ~0.3 ml of buffer S (50 mM HEPES, 150 mM NaCl, 5 mM EDTA (pH 7.4), 1% sodium dodecyl sulfate) and boiled for 1 min. Lysates were quantified using the Pierce BCA protein assay kit and subjected to SDS-PAGE (4–12% gradient precast gels, Invitrogen). Gels were transferred to PVDF membranes, incubated in TBS containing 0.1% Tween 20 (TBST) and 5% nonfat milk (w/v) for 1 h. The blots were subsequently incubated for 1 h using the appropriate primary antibody at the following antibody dilutions: 1:250 dilution for anti-AR IgG1 (Santa Cruz Biotechnology Inc., AR441), 1:1000 dilution for anti-β-spectrin II IgG1 (BD Transduction Laboratories, clone 42), 1:500 dilution for anti-nucleoporin p62 IgG2b (BD Transduction Laboratories, clone 52), 1:1000 dilution for anti-clathrin heavy chain IgG1 (BD Transduction Laboratories, clone 23), 1:500 dilution for anti-PKC δ IgG2b (BD Transduction Laboratories, clone 14), 1:5000 dilution for anti-PSA rabbit polyclonal (DakoCytomation), 1:1000 dilution for anti-protein A (Sigma, clone SPA-27), and 1:3000 dilution for anti-α-actinin-4 rabbit polyclonal (Axonera). The blots were washed three times for 5 min in TBST and incubated with either a goat anti-mouse or a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilutions in TBST for 1 h at room temperature. The blots were washed three times for 5 min in TBST, immunoreactive bands were developed and visualized using the ECL reagents kit (GE Healthcare), and the blots were exposure to Hyperfilm ECL film (GE Healthcare) for <2 min.

**Immunofluorescence**—24-h androgen-starved NTAP and NTAP-AR cells were treated with vehicle (ethanol) or androgen (10 nM R1881) for 30 min and processed for immunofluorescence imaging by labeling the DNA with DAPI, Texas Red phalloidin, and the Alexa Fluor 488 goat anti-mouse secondary conjugates (Molecular Probes) against the protein A monoclonal antibody used to detect NTAP and NTAP-AR. Geldanamycin A (1 μM/ml)-treated NTAP-AR cells were processed using the same exact protocol as detailed above.

**Silver Staining**—The appropriate amount of protein extracts was loaded onto a 4–12% SDS-PAGE gel and processed for silver staining as described previously (25).

**Hormone Treatment in LNCaP Cells**

LNCaP cells were seeded at 5 × 104 cells/cm2 into 6-well tissue culture dishes and incubated in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS) or medium B (phenol red-deficient RPMI 1640 medium supplemented with 10% charcoal-stripped FBS) for 72 h or subjected to a 48-h incubation in medium B followed by the addition of androgen (1 nM R1881) for 24 h.
Generation of Cytosolic, Nuclear, and Microsomal Protein Extracts

LNCaP cytosolic and nuclear protein extracts were generated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) as directed by the manufacturer’s protocols. NTAP and NTAP-AR cytosolic, microsomal, and nuclear protein extracts were generated as detailed previously (20). All cellular extracts were quantified using the Pierce BCA protein assay kit.

Expression Vectors, siRNA Transfections, and Luciferase Reporter Assays

Mammalian Expression Vectors—Full-length human IMAGE cDNA clones of PKC δ (IMAGE clone I.D. 5539909) and α-actinin-4 (IMAGE clone I.D. 3842046) were subcloned into the pCMV-myc (Clontech) mammalian expression vector as N-terminal Myc-tagged fusion proteins. The oligonucleotide primers (Integrated DNA Technologies) used for cloning α-actinin-4 consisted of a 5′ primer, GATCGAATTCCGATGGTGGACTACCACGCGGCGAACCAG, and a 3′ oligonucleotide primer, GAGGTTCACAAAGGAG. Nucleotide sequences of vectors encoding PKC δ were purchased and used for all transient transfection experiments. For α-actinin-4 and pCMV-myc-PKC δ were subcloned into the pCMV-myc (Clontech) mammalian expression vector as N-terminal Myc-tagged fusion proteins.

siRNA Transfections—The exact siRNA transfection conditions are detailed in the instructions provided by Dharmacon. The oligonucleotide primer pair used to clone PKC δ included the 5′ primer sequence GATCGAATTCCGATGGTGGACTACCACGCGGCGAACCAG, and a 3′ oligonucleotide primer, GATCCTCGAGTCAATCTTCCAGGAGGTGCTCGAATTTG-GGGTTCACAAAGGAG. Nucleotide sequences of vectors encoding pCMV-myc-α-actinin-4 and pCMV-myc-PKC δ were sequence-verified. These vectors were shown to induce expression of Myc-tagged α-actinin-4 and PKC δ by transient transfection in 293HEK cells. A cDNA encoding full-length sequence-verified clathrin heavy chain was purchased and used for all transient transfection experiments.

siRNA Transfections—The exact siRNA transfection conditions are detailed in the corresponding figure legends. In brief, a 21-nucleotide double-stranded siRNA duplex generated against the N terminus of the AR at nucleotides 293–312 (5′-AGGCCCATCTGTAGGGCCCA-3′) called AR1 was used for AR knockdown (26). The control siRNA duplex (non-targeting siCONTROL siRNA 1, Dharmacon) and siGENOME duplexes (Dharmacon) to AR, PKC δ, and α-actinin-4 were used for knockdown experiments. A previously characterized siRNA duplex to clathrin heavy chain (5′-GCAATGAGCTGTTT-GAAAG-3′) was used for all experiments (27). On day 0, LNCaP cells were seeded (at the specified density in the figure legends) into Falcon (BD Biosciences) 6-well tissue culture dishes and incubated in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS lacking antibiotics) for 24 h. On day 1, the cells were transfected with the corresponding siRNA at a final concentration of 50 nM using the Oligofectamine reagent according to the manufacturer’s instructions. The cells were incubated with siRNAs for a specified time. The guidelines for siRNA silencing were followed as detailed in the instructions provided by Dharmacon.

Luciferase Reporter Assays—NTAP, NTAP-AR, or CTAP-AR cells were seeded at a density of 30,000 cells/cm² into Falcon (BD Biosciences) 24-well tissue culture dishes containing medium D for 24 h. Cells were transfected in triplicate with 470 ng of total plasmid DNA/well consisting of the pMMTV-luciferase (50 ng) and pRLSV40 Renilla (Promega) (100 ng) vectors with increasing amounts of DNA encoding PKC δ, α-actinin-4, and clathrin heavy chain (10, 20, 40, 80, 160, 320, or 640 ng). The total amount of transfected plasmid DNA was held constant by the addition of pcDNA3 vectors. Positive control DNA transfections included the pGL3-luciferase (50 ng) (Promega) and pRLSV40 Renilla vectors (100 ng) plus pcDNA3 vector (320 ng). Androgens or vehicle (ethanol) was added 24 h post-transfection, and total protein lysates were measured for dual luciferase activity 48 h later according to the manufacturer’s instruction. All firefly and Renilla luciferase measurements were performed on a Veritas microplate luminometer (Turner BioSystems, Inc., Sunnyvale, CA). The mean and standard deviations were determined for all firefly luciferase values, and a pairwise Student’s t test was used to calculate significant differences (p < 0.05) between negative control transfected cells and experimental transfected cells.

Immunohistochemistry

Briefly human prostate tissue microarrays (Chemicon) were deparaffinized with toluene, rehydrated with graded alcohol washes, and subjected to antigen retrieval in a steamer for 30 min in sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in the tissues using hydrogen peroxide, and slides were incubated with normal goat serum (1:20 dilution in PBS, pH 7.4) to block nonspecific binding; this was followed by blocking endogenous biotin using the Vector staining kit (Vector Laboratories, Burlingame, CA). Slides were then incubated for 1 h with the polyclonal α-actinin-4 antibody (Axxora) in PBS at a 1:1500 dilution. Each immunohistochemical experiment was performed in parallel with a negative control staining that involved omitting the primary antibody. Slides were washed, incubated with biotinylated anti-mouse or anti-goat secondary antibody for 30 min at room temperature, and subsequently incubated with the preformed avidin-biotin-horseradish peroxidase complex. Slides were developed with the horseradish peroxidase substrate diaminobenzidine, counterstained with hematoxylin, dehydrated, and finally mounted.

RESULTS AND DISCUSSION

Development of an Androgen Receptor-expressing 293HEK Cell Line

AR has many interaction partners in mammalian cells, underscoring its multifaceted role in regulating the cellular growth and differentiation of normal and neoplastic prostate epithelial cells (11). To obtain a global view of AR-binding proteins in mammalian cells, a large scale AR purification was performed with the goal of identifying co-purifying AR-binding proteins by MS/MS (28). Unfortunately robust immunoprecipitation of endogenous AR in cancerous prostate epithelial cells using commercially available AR antibodies has been unsuccessful to date.3 As an alternative, we decided to develop a heterologous AR expression system in 293 human embryonic kidney cells capable of purifying AR and associated protein complexes in mammalian cells.

The full-length AR cDNA was cloned in-frame into the TAP module so AR was expressed as an N-terminal TAP fusion protein in 293HEK cells (Fig. 1A) (19). Neomycin-resistant 293HEK cell clones expressing the N-terminal TAP protein tag (NTAP) or N-terminal TAP-AR fusion protein (NTAP-AR) were isolated and expanded, and whole cell lysates were probed with an antibody directed against protein A to detect expression of the 25-kDa NTAP and 125-kDa NTAP-AR proteins in NTAP and NTAP-AR cell clones (Fig. 1, B, lanes 1 and 2, and C, lanes 1–5). The NTAP-N7 and NTAP-A9 cell lines expressed moderate levels of NTAP and NTAP-AR and thus were used for the following studies described below.

3 M. E. Wright, unpublished observations.
Fig. 1. Development and characterization of NTAP- and NTAP-AR-expressing cell lines. A, diagram of NTAP-AR. The AR cDNA was cloned in-frame into the pcDNA3-NTAP mammalian expression vector and expressed as an N-terminal TAP fusion protein. Protein A, red segment; TEV cleavage site, blue segment; CBP domain, green segment. B and C, stable NTAP and NTAP-AR 293HEK cell clones, respectively. Asterisks denote NTAP expression in clone N7 and NTAP-AR expression in clones A5, A7, A9, A20, and A23. Total protein extracts (10 μg/lane) from G418-resistant 293HEK clones expressing NTAP (B, clones N2 and N7, lanes 1 and 2) and NTAP-AR (C, clones A5, A7, A9, A20, and A23, lanes 1–5) were probed for protein A expression to detect NTAP and NTAP-AR expression as detailed under “Materials and Methods.” D, cellular localization of NTAP and NTAP-AR in NTAP and NTAP-AR cells. Cytosolic, microsomal, and nuclear protein extracts (10 μg/lane) were subjected to SDS-PAGE and probed with a monoclonal antibody to protein A to detect NTAP and NTAP-AR expression as detailed under “Materials and Methods.” An asterisk denotes NTAP and NTAP-AR expression in NTAP (clone N7) and NTAP-AR (clone A9) cells, respectively. E and F, androgen-mediated NTAP-AR nuclear translocation in NTAP-AR cells. In brief, NTAP-AR cells were androgen-starved in medium D for 24 h followed by the addition of vehicle (E, ethanol) or androgen (F, 10 nM R1881) for 30 min. Cells were processed for immunofluorescence imaging as detailed under “Materials and Methods.” DNA, blue stain, DAPI; NTAP-AR, green stain, goat anti-mouse Alexa Fluor 488; Actin, red stain, Texas Red phalloidin. G, androgen-mediated NTAP-AR nuclear translocation in NTAP-AR is blocked by...
**Functional Characterization of NTAP-AR Cells**

Androgens cause AR to migrate out of the cytoplasm and into the nucleus where it mediates the activation/repression of target genes in normal and neoplastic prostate epithelial cells (1). In androgen-containing growth medium, AR distributes between the cytosolic and nuclear compartments in androgen-responsive PCa cells (29,30), prompting us to probe NTAP-AR cellular localization in NTAP-AR cells. NTAP and NTAP-AR expression was probed by Western blot in crude cytosolic, microsomal, and nuclear protein extracts of NTAP and NTAP-AR cells grown in androgen-containing growth medium (growth medium supplemented with 10% FBS). Similar to AR in mammalian cells, NTAP-AR was detected in the cytosolic, membranous, and nuclear fractions in NTAP-AR cells (Fig. 1D, lanes 2, 4, and 6), whereas NTAP localized to the cytosolic fraction in NTAP cells (Fig. 1D, lanes 1, 3, and 5). Thus, NTAP-AR has a cellular localization pattern similar to that of AR in mammalian cells (6,15,29,31).

AR moves out of the cytoplasm and into the nucleus in response to androgens (30), leading us to test whether NTAP-AR shared this property. Thus, androgen-starved (24-h incubation in 10% charcoal-stripped FBS) NTAP-AR cells were treated with vehicle (30-min incubation with ethanol) or androgen (30-min incubation with 10 nM R1881), and NTAP-AR localization was monitored using indirect immunofluorescence (Fig. 1, E (vehicle) and F (androgen)). As expected, NTAP-AR localized to the cytoplasm in vehicle-treated cells (Fig. 1E), whereas it localized to the nucleus in androgen-treated cells (Fig. 1F). Thus, NTAP-AR was capable of androgen-mediated nuclear translocation similar to AR expressed in normal and neoplastic prostate epithelial cells (30). Next we tested whether this nuclear translocation process required heat shock 90-kDa protein (hsp90) chaperone activity as demonstrated for AR previously (32). Thus, androgen-starved NTAP-AR cells were pretreated with geldanamycin A (45 min), a potent inhibitor of hsp90 (30), and subsequently challenged with androgen (Fig. 1G). Geldanamycin A effectively blocked androgen-mediated NTAP-AR nuclear translocation as NTAP-AR displayed a pronounced, perinuclear staining pattern (Fig. 1G), suggesting that hsp90 activity was required for NTAP-AR nuclear translocation in NTAP-A9 cells as shown for AR in mammalian cells (34).

Next we tested whether NTAP-AR could mediate androgen-dependent transcription similar to AR in mammalian cells (1). Thus, NTAP and NTAP-AR cells were transfected with the AR-responsive mouse mammary tumor virus-luciferase (pM-MTV-luc) reporter and treated with androgen (Fig. 1H). Firefly luciferase activity was readily detectable in NTAP and NTAP-AR cells transfected with the constitutive pGL3-luciferase reporter (Fig. 1H, columns 1 and 2). In contrast, transfection of the pMMTV-luc reporter into androgen-starved NTAP and NTAP-AR cells resulted in minimal firefly luciferase activity respectively (Fig. 1H, column 3). However, the addition of androgen caused an ~10-fold increase in luciferase activity in NTAP-AR cells relative to NTAP cells (Fig. 1H, column 4). Taken together, the above results show that NTAP-AR retains many of the biochemical properties of AR as a SHR (1), and thus the NTAP-AR cell line represents a novel heterologous expression system for studying AR function in mammalian cells.

**Purification of NTAP and NTAP-AR Protein Complexes**

A major goal of this analysis was to use a large scale protein purification strategy coupled to MS/MS to identify novel AR-binding proteins that influence AR function in mammalian cells. Recently the ICAT method, a quantitative mass spectrometry approach that incorporates isotopic protein labels into cysteine-containing proteins for subsequent MS protein identification and quantification (35), was used to identify and quantify affinity-purified protein complexes isolated from yeast and mammalian cells (25,36). For example, the yeast polymerase (pol) II transcription initiation complex was comprehensively characterized through the incorporation of isotopic labels (ICATs) into an affinity-purified pol II protein complex that guided the identification of bona fide pol II protein components relative to nonspecific, co-purifying proteins by their enriched ICAT protein abundance ratios measured in the mass spectrometer (25,36). We used a similar ICAT strategy to identify AR-binding proteins by comparing the protein abundance ratios of NTAP and NTAP-AR protein complexes (Fig. 24). Contaminants are expected to be present in control (NTAP alone) and NTAP-AR purifications in equal amounts (ICAT ratios of 1). By contrast, specific interactions should be significantly enriched in the NTAP-AR sample (ICAT ratios >1).

The standard TAP protocol utilizes a two-step purification strategy (37). However, to enrich for both high and low affinity AR-binding protein complexes the NTAP and NTAP-AR protein complexes were purified after a single purification step over an IgG-Sepharose column. In brief, crude cytosolic and nuclear protein fractions derived from androgen-stimulated (24-h incubation in 100 nM R1881) NTAP and NTAP-AR cells...
were purified in parallel over an IgG-Sepharose column to enrich for NTAP- and NTAP-AR-binding proteins, respectively (Fig. 2A). Although NTAP completely bound to the IgG-Sepharose column (Fig. 2B, see lane 2), NTAP-AR binding was incomplete in the cytosolic extracts of NTAP-AR cells (Fig. 2B, see lane 3), suggesting that the binding capacity of the column was exceeded or a small amount of NTAP-AR was unable to bind the IgG-Sepharose column. TEV-mediated cleavage of column-purified NTAP-AR effectively eluted the calmodulin binding peptide (CBP)-AR fusion protein from the column (Fig. 2, lane 6). Noticeably two to three discrete immunoreactive bands were purified after TEV cleavage (Fig. 2, lanes 5 and 6), demonstrating that AR partially degraded during the purification process. Nuclear protein extracts isolated from NTAP and NTAP-AR cells were subjected to the same exact purification procedure (Fig. 2C). In contrast to the cytosolic NTAP purification, NTAP was absent in the nuclear extracts of NTAP-expressing cells (Fig. 2C, lane 1), whereas NTAP-AR was purified from the nuclear extracts of NTAP-AR-expressing cells (Fig. 2C, lanes 1 and 3). Similar to the cytosolic purification of NTAP-AR, TEV effectively eluted the CBP-AR fusion protein from the IgG-Sepharose column (Fig. 2C, lane 6). The cytosolic and nuclear TEV-eluted NTAP and NTAP-AR extracts were subsequently subjected to the ICAT method as described below.

Identification and Quantification of NTAP and NTAP-AR Protein Complexes

To identify cytosolic and nuclear AR-binding proteins in NTAP-AR-expressing cells, equal amounts of cytosolic or nuclear TEV-eluted NTAP and NTAP-AR protein extracts were compared with each other using the ICAT method (Fig. 2A). In brief, all cysteine-containing proteins in NTAP protein extracts were labeled with ICAT reagents and subjected to reverse-phase liquid chromatography followed by mass spectrometric analysis. The resulting protein complexes were then visualized using Western blot analysis with monoclonal antibodies to protein A and AR as described under “Materials and Methods.”

Fig. 2. Purification strategy of NTAP and NTAP-AR protein complexes using the ICAT method. A, in brief, protein extracts (cytosolic and nuclear) isolated from 24-h androgen-treated (100 nm R1881) NTAP and NTAP-AR cells were purified over an IgG-Sepharose column as detailed under “Materials and Methods.” The columns were washed and incubated with the TEV protease to elute proteins for analysis using the ICAT method as detailed under “Materials and Methods.” B and C, Western blot expression of cytosolic and nuclear purified NTAP and NTAP-AR protein complexes, respectively. B, cytosolic purification: lanes 1–4, 20 μg of protein; lanes 5 and 6, 2 μg of protein. C, nuclear purification: lanes 1–4, 20 μg of protein; lanes 5 and 6, 2 μg of protein. Protein extracts were subjected to SDS-PAGE and probed with monoclonal antibodies to protein A and AR as described under “Materials and Methods.”
were labeled with the light \((d_0)\) ICAT reagent, whereas NTAP-AR protein extracts were labeled with the heavy \((d_8)\) ICAT reagent. The samples were combined, proteolyzed with trypsin, and separated into 30 fractions by strong cation exchange. Labeled cysteine-containing peptides were captured with avidin and analyzed by microcapillary LC-MS/MS. These experiments led to the quantification of 211 and 300 proteins in the cytosolic and nuclear fractions, respectively (Table I) (ProteinProphet scores \(\geq 0.95\)) (Fig. 3 and Supplemental Panels 1A and 1B) (23). A total of 421 unique quantified ICAT-labeled proteins were detected in the cytosolic and nuclear protein fractions with 90 proteins common between both fractions (Fig. 3A and Supplemental Panel 2). More importantly, ICAT-labeled AR peptides showed a 2.8- and 5-fold enrichment in the cytosolic and nuclear protein fractions, respectively (Supplemental Panel 2), demonstrating that the purification scheme successfully enriched for AR and possibly other co-purifying AR-binding proteins.

**Table I**

|                  | Cytosolic | Nuclear |
|------------------|-----------|---------|
| Identified ICAT-labeled proteins | 252       | 399     |
| Quantified ICAT-labeled proteins   | 211       | 300     |
| Proteins with ICAT ratio \(\geq 1.5\) | 87        | 124     |

**Gene Ontology Classification of Proteins**

Gene ontology annotation was used to functionally assign the 421 proteins based upon their biological process and molecular function (38). This would allow us to accomplish the following goals. First, it would provide a global view of the various protein classes represented by the 421 ICAT-labeled proteins. Second, an arbitrary but biologically determined ICAT enrichment ratio (e.g. \(d_0:d_8\) of 1.3, 1.5, etc.) could be assigned to the proteins to determine whether specific protein modules were enriched after AR purification. The 421 ICAT-labeled proteins were uploaded into Cytoscape, a powerful visualization program for integrating biomolecular interaction networks (39), and the Cytoscape plug-in Bingo was used to identify statistically \((\leq 0.05)\) overrepresented protein modules contained in the 421 ICAT-labeled protein dataset (40). Protein modules comprising the biological processes of RNA processing, transport, cytoskeletal organization, and protein folding were found to be statistically overrepresented in our dataset (Supplemental Panels 3A and 3B). Protein modules associated with the molecular function of cytoskeletal binding, protein transport, and RNA binding were also statistically overrepresented (Supplemental Panels 4A and 4B). In summary, these results demonstrate that NTAP and NTAP-AR purification led to the enrichment of specific protein modules.

The enriched protein modules may simply reflect the high level of abundance these proteins represent in both the cytosolic and nuclear protein extracts that nonspecifically bound to the column during the purification process. Alternatively the observed protein modules may represent functional AR interactions that were enriched during AR purification. To further
explore whether the quantified proteins shared any overlap with known AR-binding proteins all ICAT-labeled proteins were compared with the 118 previously annotated AR-binding proteins at the McGill Androgen Receptor Gene Mutations and Human Protein Reference Databases (HPRD) (Supplemental Panel 5) (18, 41). Although this number probably underestimates the total number of AR-binding proteins in the literature, it provides a rough estimate of AR-binding proteins published to date. The 421 ICAT-labeled protein dataset contained 14 of the 118 (~12%) known AR-binding proteins, demonstrating a low level of overlap between the datasets (Fig. 3B and Supplemental Panel 6A). Next putative AR-binding proteins were defined as any protein that contained an ICAT protein enrichment ratio equivalent to or greater than 1.50 (both cytosolic and nuclear d0/d8 ICAT ratios) because Dna J homolog subfamily C member 7, a chaperone that binds glucocorticoid receptor (42), contained a 1.50 ICAT ratio (Supplemental Panels 1A and 1B). This 1.50-fold ICAT protein enrichment value reduced the 421 ICAT-labeled protein dataset to 181 putative AR-binding proteins (Fig. 3C, Table II, and Supplemental Panel 7). Importantly this group of 181 proteins contained 12 of the 118 (~10%) previously reported AR-binding proteins annotated in the McGill and HPRD databases (Fig. 3C, Table II, and Supplemental Panel 6B). This subset of 12 proteins represented a well studied group of chaperones and transcriptional regulators known to modulate AR function in PCa cells (Table II and Supplemental Panel 6B) (11). Cytoscape and Bingo analyses revealed that the 181 putative AR-binding proteins overrepresented the biological processes of RNA processing, protein folding, and cytoskeletal biogenesis (Supplemental Panels 8A and 8B), whereas the molecular functions of RNA binding, protein binding, and cytoskeletal protein-binding proteins were enriched in this dataset (Fig. 4 and Supplemental Panel 8C). The low degree of overlap between the putative AR-binding proteins reported here and AR-binding proteins annotated at the McGill and HPRD databases is probably related to the observation that many reported AR-binding proteins were detected using a specific AR domain (e.g. ligand-binding or DNA-binding domain) in a Y2H or GST fusion protein interaction assay (17, 18, 41). Thus, domain-specific AR-binding proteins in the Y2H or GST-protein interaction assay may share little overlap to AR-binding proteins detected with the intact NTAP-AR molecule described in this study.

In depth analysis of putative AR-binding proteins in the cytosolic and nuclear ICAT datasets uncovered a group of proteins with incongruent cytosolic and nuclear ICAT ratios with respect to NTAP-AR purification (Supplemental Panel 2). For example, proteins involved in cytoskeletal organization were increased in the nuclear fraction (d0/d8 ICAT ratio of ≥1.5), and their levels were dramatically reduced in the cytosolic fraction (d0/d8 ICAT ratios of =0.66) respectively (Supplemental Panel 2). Several scenarios could explain these incongruent ICAT ratios relative to NTAP-AR purification. However, both outcomes are based upon the premise that NTAP-AR binds to proteins in the cytosol and translocates these molecules into the nucleus in response to androgen stimulation, whereas NTAP remains localized to the cytoplasm (Fig. 5). For example, the first scenario may involve a direct association of the CBP domain located in the TAP module to cytoskeletal proteins mediated through a ternary complex interaction with endogenously expressed calmodulin (CaM). CaM is known to bind filamin A and also interact with other cytoskeletal components (43, 44), and under the purification conditions used, TEV-mediated cleavage of NTAP and NTAP-AR affinity-purified extracts could facilitate the release
of the CBP domain and associated cytoskeletal proteins from the IgG-Sepharose column. Cytoskeletal proteins would be enriched in TEV-eluted NTAP cytosolic protein extracts relative to TEV-eluted NTAP-AR cytosolic protein extracts because this same group of cytoskeletal proteins would have migrated into the nucleus through their physical interaction with NTAP-AR in response to androgen (Fig. 5A). Conversely TEV-eluted nuclear protein extracts would be enriched for cytoskeletal proteins in NTAP-AR cells relative to NTAP cells because the cytoskeletal proteins in NTAP cells would remain in the cytosol in response to androgen (Fig. 5B). During the second scenario, NTAP-AR could bind CaM through a direct physical interaction with AR as noted previously (45). Regrettably the results cannot discern either scenario. Previous studies have demonstrated a physical interaction between AR and the cytoskeletal proteins filamin A and gelsolin (46), and thus we tend to believe the cytoskeletal protein enrichments were mediated through a direct physical interaction with NTAP-AR. Filamin A and gelsolin are known co-regulators of AR-mediated transcription (47), and both proteins were enriched in the nuclear compartment with NTAP-AR (Supplemental Panel 2), suggesting that they co-migrated into the nucleus in NTAP-AR cells (Fig. 5). The different protein modules that were enriched during AR purification suggest that AR interacts with multiple protein complexes as it would be difficult to imagine AR binding to these proteins in a simultaneous manner as a megaprotein complex. Thus, congruent and incongruent ICAT ratios equal to or greater than 1.50-fold ($d_0:d_8 \geq 1.50$ and $d_0:d_8 \leq 0.66$) relative to NTAP-AR purification will be considered putative AR-binding proteins from this point on in the analysis.
**Functional Classes of Putative AR-binding Proteins**

Based upon the large group of proteins that bind AR (17, 41), it is reasonable to predict that AR will bind to protein complexes that localize to the cytosol and nucleus as well as bind to specific protein complexes that localize to either cellular compartment exclusively as it moves between the cytosol and nucleus in response to androgens. The enrichment of a specific protein module that co-purified with NTAP-AR could suggest a functional interaction between AR and this group of proteins. This prompted us to examine the enrichment of protein modules that co-purified with NTAP-AR in the cytosolic and nuclear protein fractions. Ideally quantification of all proteins in both the cytosolic and nuclear protein fractions would provide a comprehensive picture of all AR-binding proteins in both cellular compartments. However, this scenario is highly unlikely for the following two reasons. First, AR probably binds to specific subsets of proteins that localize exclusively to either the cytosolic or nuclear compartments, respectively, and thus protein quantification would be unavailable for this category of proteins. Second, it would require extensive MS sequence coverage of all co-purified, cysteine-containing proteins in the cytosolic and nuclear fractions. Regrettably high sequence coverage was not available for the majority of proteins quantified in this study (Supplemental Panels 1A and 1B). Despite these limitations, all quantified proteins were sorted into two categories, an “overlap” category (Supplemental Panel 2), which contained all proteins quantified in both the cytosolic and nuclear protein fractions, and a “non-overlap” category (Supplemental Panel 9), which contained all proteins quantified in either the cytosolic or nuclear protein fractions, respectively. A total of 90 proteins belong to the overlap category (Supplemental Panel 2), which upon closer inspection revealed a group of cytoskeletal proteins (e.g. FLNA, SPTBN1, ACTN4, and EPB41L2) that were specifically enriched (d_c:d_n ≥ 1.50 and d_c:d_n ≤ 0.66) and co-purified with NTAP-AR, suggesting a physical association with AR. Multiple studies have shown how androgens can induce rapid actin cytoskeleton changes in epithelial cells (48, 49). This rapid transcription-independent activity is thought to occur through a membrane-associated AR (50). Our results give support to these prior observations and suggest that cytoskeletal proteins may have a more involved role in AR signaling than previously expected. To identify other putative AR-containing protein modules, all enriched proteins were uploaded into Cytoscape and analyzed using the Bingo plug-in as detailed above. The biological processes of RNA processing (e.g. HNRPM, SNRPD3, CPSF6, RBM25, and DDX39) and protein folding (e.g. FKBP4, BAG2, and ST13) were overrepresented in the cytosolic and nuclear fractions, suggesting a functional interaction between these specific protein modules and AR in the cytosolic and nuclear compartments, respectively (Supplemental Panels 8A and 8B). For example, SHRs are known to couple transcription and RNA splicing through their interactions with the RNA-binding proteins p72 and coactivator activator (51, 52). Also proteins involved in protein folding, better known as chaperones, are known regulators of SHR maturation and SHR-mediated transcription (8). Calreticulin (CALR), which can also bind SHRs and function as a negative regulator of SHR-mediated transcription in mammalian cells (53) (Supplemental Panel 7), was enriched in this fraction. Interestingly the enrichment of vesicular transport proteins adaptor-related protein complex 1 (AP1G1) and clathrin heavy chain polypeptide (CLTC) was unexpected given that their role in AR signaling is not well documented (54).

Inspection of the non-overlap category (331 proteins) re-
revealed a group of enriched proteins that co-purified with NTAP in the cytosolic (e.g., P15RS, DCXR, and My016) and nuclear (e.g., VDAC2, CCT4, UQCRCl1, and UQCRH) protein fractions (Supplemental Panel 9). As described above, these putative NTAP-binding proteins may actually represent putative NTAP-AR-binding proteins that have undergone androgen-mediated cytoplasmic/nuclear translocation in NTAP-AR cells (Fig. 5). A more definitive explanation would be forthcoming if ICAT values were available for all proteins in both the cytosolic and nuclear fractions, respectively. Despite this limitation, the non-overlap category did contain co-purified proteins that were enriched with NTAP-AR in the cytosolic fraction (Supplemental Panel 9). This group included the chaperones 70-1 (HSPA1A) and 90α (HSPCA), which as discussed above, are important regulators of SHR activity in mammalian cells (32). β-Catenin-1 (CTNNB1) was also enriched in the cytosolic fraction (Supplemental Panel 9); it is a known co-regulator of AR-mediated transcription in PCA cells (55). The nuclear protein fraction also contained enriched RNA-binding and RNA processing proteins (e.g., ASCC3L1, RNPC2, CPSF3, HNRPD, HNRPDL, and HNRPR) (Supplemental Panel 9), thus supporting the functional link between AR and mRNA splicing (51). As noted above, the NTAP-AR-co-purifying endosomal and vesicular transport proteins were also enriched in the nuclear fraction (e.g., HIP1, SNX9, GOLGA5, SYPL1, and AP2A2). Importantly huntingtin-interacting protein 1 (HIP1), which binds to clathrin and facilitates clathrin-mediated endocytosis (54), can induce tumorigenesis when overexpressed in epithelial cells and is frequently overexpressed in advanced PCA (56, 57). Recently, HIP1 was shown to bind AR and function as a co-activator of AR-mediated transcription in PCA cells (54). In summary, these results demonstrate that specific protein modules were enriched after NTAP-AR purification in mammalian cells, suggesting a functional interaction between these molecular pathways and AR in normal and cancerous prostate epithelial cells.

**Functional Characterization of Putative AR-binding Proteins**

**Effect of Androgen upon the Expression and Localization of Putative AR-binding Proteins in Prostate Cancer Cells**—The enrichment of specific protein modules with NTAP-AR prompted us to study what role, if any, a single enriched protein had upon AR signaling in PCA cells. Androgens can induce gross changes in cell morphology, and thus we wanted to determine whether androgens had any effect upon the cellular localization of targeted proteins. We decided to characterize AR-co-enriched proteins that were components of the cytoskeleton and endosome in mammalian cells (Supplemental Panels 1A and 1B). This group of proteins included β-spectrin II, α-actinin-4, and clathrin heavy chain. Their relative expression levels were probed by Western blot in the androgen-responsive human LNCaP prostate cancer cell line. Crude cytosolic and nuclear protein extracts were isolated from LNCaP cells that were grown in standard (phenol red-deficient RPMI 1640 medium + 10% FBS), androgen-depleted (AD-treated) (phenol red-deficient RPMI 1640 medium + 10% charcoal-stripped FBS), or androgen-stimulated (AS-treated) (phenol red-deficient RPMI 1640 medium + 10% charcoal-stripped FBS for 48 h + 1 nm R1881 for 24 h) growth medium (Fig. 6, A–E). We initially decided to probe AR in the cytosolic and nuclear fractions to demonstrate how androgens can influence protein expression and localization accordingly. The authenticity of cytosolic and nuclear protein extracts was confirmed by probing nucleoporin p62 expression in fractionated LNCaP cells (Fig. 6A, lanes 1–6). As shown previously (29), AR localized to the cytosolic and nuclear fractions in cells that were grown in standard growth medium (Fig. 6B, lanes 1 and 2). In contrast, AR levels increased in the cytosolic fraction of AD-treated cells (Fig. 6B, lanes 1 and 3), whereas androgenic stimulation caused AR levels to increase in the nuclear fraction of AS-treated cells (Fig. 6B, lanes 2 and 6). These results are in agreement with the known effects of androgens upon AR expression and localization in PCA cells (30). Next the levels and localization pattern of β-spectrin II, both an essential structural component in the cytoskeleton and scaffolding adaptor of transforming growth factor-β-mediated signaling by Smad proteins (58), was probed in LNCaP cells. Based upon its central role in the cytoskeleton, β-spectrin II was primarily localized to the cytosolic fraction in cells grown in standard growth medium (Fig. 6C, lanes 1 and 2). Androgen depletion had no obvious effect on β-spectrin II levels in AD-treated cells (Fig. 6, lanes 1 and 3 and lanes 2 and 4), whereas androgen stimulation caused a slight increase in nuclear β-spectrin II levels in AS-treated cells (Fig. 6C, lanes 2 and 6). Next we probed α-actinin-4 and clathrin heavy chain expression in LNCaP cells accordingly. Both α-actinin-4 and clathrin heavy chain were distributed equally between the cytosolic and nuclear fractions in cells grown in standard growth medium (Fig. 6D, E, lanes 1 and 2). Androgen depletion and androgenic stimulation caused a slight increase in nuclear α-actinin-4 levels in the cytosolic and nuclear fractions of AD- and AS-treated cells (Fig. 6D, lanes 3 and 4 and lanes 5 and 6). Interestingly androgen depletion caused clathrin heavy chain levels to increase dramatically in the nuclear fraction of AD-treated cells (Fig. 6E, lanes 2 and 4). This increase in clathrin heavy chain expression was also observed in the cytosolic and nuclear fraction of AS-treated cells, respectively (Fig. 6E, lanes 1 and 5). Clathrin heavy chain was characterized previously as a direct gene target of AR-mediated transcription (59), offering a mechanism for the increased expression of clathrin heavy chain in AS-treated LNCaP cells. In brief, these results suggest that androgens can effect β-spectrin II, α-actinin-4, and clathrin heavy chain expression and or localization in LNCaP cells and that these molecules may be components of androgen/AR action in normal and neoplastic prostate epithelial cells.
Effect of α-Actinin-4, PKC δ, and Clathrin Heavy Chain Expression siRNA-mediated Knockdown upon AR and PSA Expression in LNCaP Cells—Next α-actinin-4 and clathrin heavy chain were targeted for siRNA-mediated knockdown to determine whether their expression had any effect upon AR expression levels or AR-mediated transcription in LNCaP cells.

Fig. 6. Androgen-mediated protein expression changes in the cytosolic and nuclear compartments of LNCaP cells. A–E, Western blot analysis of p62 nucleoporin (A), AR (B), β-spectrin II (C), α-actinin-4 (D), and clathrin heavy chain (E) expression in LNCaP cytosolic (c; lanes 1, 3, and 5) and nuclear (n; lanes 2, 4, and 6) protein extracts. In brief, cytosolic and nuclear protein extracts isolated from LNCaP cells under three experimental conditions that included a 72-h incubation in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS), medium B (androgen-depleted, phenol red-deficient RPMI 1640 medium supplemented with 10% charcoal-stripped FBS) or a 48-h incubation in medium B followed by the addition of androgen (androgen-stimulated, 1 nM R1881) for 24 h. 4 μg of the corresponding total protein extracted was subjected to SDS-PAGE and probed with the corresponding antibodies as detailed under “Materials and Methods.” F, silver stain of 4 μg of cytosolic and nuclear protein extracts used for the Western blots (A–E) to demonstrate equivalency of protein loading. Data are representative of at least three independent experiments.
cells. Additionally PKC δ, which is a common component of multiple cell surface receptor signaling cascades in mammalian cells (60), was also targeted for siRNA-mediated knockdown because it was enriched in the nuclear fraction with NTAP-AR (Supplemental Panel 1B). Targeting clathrin heavy chain, PKC δ, and α-actinin-4 for siRNA-mediated knockdown dramatically reduced their expression in transfected LNCaP cells accordingly (Fig. 7, A–C). We initially probed AR expression in siRNA-transfected LNCaP cells in standard growth medium (Fig. 7D, lanes 1–5). As expected, AR knock-
down decreased AR expression in targeted cells relative to control knockdown cells (Fig. 7D, upper panel, lanes 1 and 2), whereas AR levels were relatively unchanged in clathrin heavy chain, PKC δ, and α-actinin-4 knockdown cells, respectively (Fig. 7D, upper panel, lanes 1–3, 4, and 5). Although AR levels remained unchanged in these knockdown cells, we wanted to test whether their knockdown would perturb PSA expression in LNCaP cells because PSA represents a well characterized downstream target of AR-mediated transcription in PCa cells (61). As anticipated, PSA levels decreased dramatically in AR knockdown cells (Fig. 7D, lower panel, lanes 1 and 2). Unexpectedly PSA levels were noticeably reduced in clathrin heavy chain knockdown cells (Fig. 7D, lower panel, lanes 1 and 3). PSA levels were also slightly reduced in α-actinin-4 knockdown cells and remained relatively unchanged in PKC δ knockdown cells (Fig. 7D, lower panel, lanes 1, 4, and 5). Interestingly the reduced PSA expression in clathrin heavy chain knockdown cells did not correlate with a decrease in AR expression as noted in AR knockdown cells (Fig. 7D, upper panel, lanes 2 and 3), thus ruling out the possibility that the decrease in PSA expression was mediated by a reduction in AR levels. These experiments suggest that clathrin heavy chain and α-actinin-4 are required for normal PSA expression in LNCaP cells and suggest that these molecules may facilitate AR-mediated transcription in normal and neoplastic prostate epithelial cells.

Next we tested whether clathrin heavy chain, PKC δ, and α-actinin-4 knockdown had any effect upon AR expression or AR-mediated transcription following the readdition of androgen to androgen-starved LNCaP cells. For example, androgen starvation causes AR to accumulate in the cytosol of mammalian cells (4, 32). After the addition of androgen, AR is proteolytically processed by the proteasome while it transits into the nucleus to activate and or repress target downstream genes (8, 62). As expected, AR levels were noticeably decreased in androgen-starved AR knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 1–4). According to PSA expression upon the readdition of androgen in AR knockdown cells was blocked (Fig. 7E, lower panel, lanes 1–4), thus confirming the AR-dependent nature of PSA expression in LNCaP cells. Interestingly, AR levels also decreased in androgen-starved clathrin heavy chain knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 1 and 5). Although AR levels were noticeably higher in androgen-treated clathrin knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 2 and 6), PSA levels were somewhat decreased in these cells relative to control cells (Fig. 7E, lower panel, lanes 2 and 6). Similarly although a slight reduction in AR levels was observed in androgen-starved PKC δ knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 1 and 7), AR levels were noticeably increased in androgen-treated PKC δ knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 2 and 8). Interestingly, this coincided with an incongruent decrease in PSA expression relative to control cells (Fig. 7E, lower panel, lanes 2 and 8). In contrast, although AR levels were relatively unchanged in androgen-starved α-actinin-4 knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 1 and 9), both AR and PSA levels were noticeably increased in androgen-treated α-actinin-4 knockdown cells relative to control knockdown cells (Fig. 7E, upper panel, lanes 2 and 10, and lower panel, lanes 2 and 10). Collectively these knockdown experiments suggest that androgen-starved LNCaP cells require clathrin heavy chain, PKC δ, and α-actinin-4 expression for normal AR and PSA expression in response to androgens. Although gross fluctuations in AR and PSA expression were not detected by these knockdown studies, perturbations in clathrin heavy chain, PKC δ, and α-actinin-4 expression did induce moderate changes in AR expression and AR-mediated transcription in LNCaP cells. For example, clathrin heavy chain and PKC δ may act to either stabilize AR synthesis or inhibit AR degradation in the absence of androgen, thus causing AR levels to decrease in clathrin heavy chain and PKC δ knockdown cells. Alternatively clathrin heavy chain and PKC δ could also promote AR degradation in the presence of androgen because AR ubiquitination and proteolytic processing are critical steps for its nuclear translocation and transcriptional activity in PCa cells (62). In contrast, α-actinin-4 expression had no obvious effect on AR levels in androgen-starved LNCaP cells, whereas α-actinin-4 expression was necessary for normal PSA expression in response to androgenic stimulation. At the molecular level, α-actinin-4 may target AR for degradation and or antagonize AR synthesis upon the addition of androgen. Lastly α-actinin-4 may negatively regulate AR-mediated transcription; this could explain why PSA levels were slightly increased after androgenic stimulation in α-actinin-4 knockdown cells. Alternatively α-actinin-4 may promote PSA degradation in LNCaP cells through a post-transcriptional mechanism. Studies to test these new hypotheses are forthcoming. In summary, these results suggest that clathrin heavy chain, PKC δ, or α-actinin-4 expression can influence androgen/AR signaling in PCa cells.

Effects of PKC δ, α-Actinin-4, and Clathrin-Heavy Chain Overexpression upon AR-Mediated Transcription—The above studies prompted us to test whether clathrin heavy chain, PKC δ, or α-actinin-4 overexpression could increase or decrease AR-mediated transcription similarly to known AR coregulators. Another heterologous 293HEK AR expression cell line called CTAP-AR was developed that expressed AR with a C-terminal TAP chitin-binding domain tag (Fig. 8A). The CTAP-AR cell line provided us with another AR expression system to test whether clathrin heavy chain, PKC δ, or α-actinin-4 could function as co-regulators of AR-mediated transcription in mammalian cells. CTAP-AR cells were highly responsive to androgens as luciferase activity (~100-fold induction) was readily detected in cells transfected with the pMMTV-luc reporter after the addition of androgen (Fig. 8B),
Thus demonstrating the androgen-dependent transcriptional activity of CTAP-AR. Therefore, increasing amounts of cDNA encoding clathrin heavy chain, PKC δ (PRKCD), and α-actinin-4 (ACTN4) were transiently co-transfected with the pMMTV-luc reporter and stimulated with androgen, and their effects upon CTAP-AR-mediated transcription were measured accordingly (Fig. 8, C–E). We noticed that a small amount of transfected α-actinin-4 cDNA (10 ng) slightly increased CTAP-AR-mediated transactivation (Fig. 8C), whereas a dose-dependent increase in transfected α-actinin-4 cDNA progressively decreased CTAP-AR-mediated transactivation (Fig. 8C). Similarly the smallest quantity of transfected PKC δ cDNA (10 ng) slightly increased CTAP-AR transactivation (Fig. 8D), whereas a dose-dependent increase in transfected PKC δ cDNA steadily decreased CTAP-AR-mediated transactivation (Fig. 8D). In contrast, only the highest quantity (320 ng) of transfected clathrin heavy chain cDNA was able to increase CTAP-AR-mediated transactivation (Fig. 8E). To show that clathrin heavy chain could clearly increase CTAP-AR-mediated transcription, a higher quantity (640 ng) of clathrin heavy chain cDNA was transfected into CTAP-AR cells; this led to a ~5-fold increase in CTAP-AR-mediated transcription accordingly (Fig. 8F).

Together these co-reporter assays demonstrate that α-actinin-4, PKC δ, and clathrin heavy chain can function as co-regulators of AR-mediated transcription in mammalian cells. Interestingly although α-actinin-4 and PKC δ displayed expression-dependent co-activator and co-repressor activity toward CTAP-AR-mediated transcription, only clathrin heavy chain displayed strong co-activator activity during CTAP-AR-mediated transcription under the experimental conditions.
Androgen Receptor Protein Modules

![Fig. 9. Immunohistochemical validation of α-actinin-4 expression in prostate tissues. Immunohistochemical staining with anti-α-actinin-4 antibody (A, C, E, G, and I) and hematoxylin and eosin (H & E) staining of serial corresponding sections (B, D, F, H, and J). A and B show normal glands with strong α-actinin-4 staining in the basal cells of the glands and much weaker staining in the luminal glands (40×). C and D show benign prostatic hyperplasia with some atypical features and apparent loss of strong α-actinin-4 staining in the basal cells of the prostatic glands. E and F show Gleason pattern 3 glands (40×; Gleason score 5 (2 + 3) cancer) with multiple nuclei stained positive for α-actinin-4 (arrows) where the glands are well defined with well formed luminal architecture. G and H show Gleason type 4 glands (40×; Gleason score 7 (3 + 4) cancer) showing very minimal or absence of staining in the nuclei (arrow). Note that glandular architecture is less well defined compared with Gleason 3 glands in E and F. I and J are 60× images of Gleason pattern 5 prostate cancer (Gleason score 10 (5 + 5)) where nuclei show very minimal staining with the anti-α-actinin-4 antibody.](Image)

The co-repressor activity detected by PKC δ overexpression compliments a previous study that showed how RACK1, a scaffolding molecule that binds to specific isoforms of protein kinase C and AR (63, 64), was a negative regulator of AR-mediated transcription when overexpressed in prostate cancer cells (65). PKC δ levels and activity may determine its role as a co-regulator of AR-mediated transcription in prostate cancer cells. In relation to PCa, PKC δ is a component of the interleukin-6 (IL-6) receptor complex (66), and IL-6 signaling is known to increase during advanced stage PCa (67). Multiple studies suggest that IL-6 and AR signaling pathways are synergistic or antagonistic in PCa cells (68–72). A recent study has shown that androgens promote a physical association between AR and glycoprotein 130 (gp130), an essential component of the IL-6 receptor signaling complex (66), that acts to inhibit IL-6 signaling in PCa cells (73). Our results complement this study and suggest that PKC δ may represent another component of the AR-gp130 protein complex. AR may inhibit PKC δ activity through its direct binding and or sequestering of PKC δ away from the gp130 signaling complex. Future exploration of these new hypotheses is greatly anticipated. Interestingly, α-actinin-4 and clathrin heavy chain, both regulators of endosomal functions and cell surface receptor cycling (74, 75), were capable of modulating CTAP-AR-mediated transcription. The endosomal pathway is known to play an important role during growth factor signaling (e.g. epidermal growth factor) (74). However, its role in regulating SHR-mediated transcription is only now being realized as HIP1, a critical component of the endocytotic machinery, was recently shown to modulate AR-mediated transcription (54). Future studies to explore how the endosome can modulate AR signaling in PCa cells are forthcoming.

α-Actinin-4 Expression in Prostate Tissues

To date, α-actinin-4 is best known as a cytosolic protein involved in regulating endocytosis and actin-mediated cytoskeletal processes in epithelial cells (74, 75). However, α-actinin-4 has been localized exclusively to the nucleus in several human cancerous epithelial cell lines (76) and has also been shown to undergo nuclear translocation in human foreskin keratinocytes upon the addition of wortmannin, a well known phosphatidylinositol 3-kinase inhibitor (17, 76). Most interestingly, loss of nuclear α-actinin-4 expression correlated with invasive metastatic breast cancer as these patients were shown to display a poorer survival rate (76). To explore α-actinin-4 expression and localization in normal and cancerous human prostate tissue samples, a human tissue microarray slide was subjected to α-actinin-4 immunohistochemical staining (Fig. 9, A–C). As shown in Fig. 9, A and B, α-actinin-4 is predominantly expressed in the basal layer of normal prostate epithelium, and this staining pattern is lost in the benign prostatic hyperplasia (Fig. 9, C and D). Interestingly the nuclei of the luminal epithelial cells in Gleason pattern 3 cancerous prostate glands were prominently stained (Fig. 9, E and F). However, this nuclear staining pattern was replaced by a prominent cytosolic staining pattern in the poorly defined glandular structures of advanced PCa cells (Fig. 9, G and H). Consistent with this observation, α-actinin-4 strongly stained the cytosol and was completely absent in the nucleus of advanced PCa cells (Fig. 9, I and J). These results indicate that α-actinin-4 protein expression and localization may reflect specific biological states during the prostate tumorigenesis process as summarized in Table III. Future studies to investigate the molecular mechanisms of α-actinin-4 localization and function in advanced stage PCa cell lines and tissues will help to elucidate its role in the growth and maintenance of advanced PCa.
We have developed a heterologous AR expression system in the 293HEK cell line that recapitulates AR SHR activity in PCa cells (2). This system identified putative AR-binding proteins in the cytosolic and nuclear extracts using the ICAT method (25, 35). Established AR-binding proteins (e.g. chaperones) that influence AR signaling in normal and neoplastic prostate epithelium were detected. More importantly, several groups of functionally related proteins were specifically enriched during AR purification (e.g. cytoskeletal, RNA binding, protein transport), suggesting a functional link between these protein networks and AR in vivo. AR binds to a large, functionally diverse group of proteins, making it difficult to fully understand the multiple activities of AR during the growth and differentiation of normal and neoplastic prostate epithelial cells in vivo. This study represents the first attempt at constructing a large scale AR-protein interaction map in mammalian cells using quantitative mass spectrometry. Interestingly the putative AR-binding proteins detected in this study showed little overlap to AR-binding proteins annotated at the McGill and HPRD databases. Many studies detected AR-binding proteins using truncated AR domains in the Y2H or GST-protein interaction assays (e.g. ligand-binding domain and DNA-binding domain) (18). The multidomain structure of AR makes it difficult to study protein-protein interactions to intact AR by these methods. The NTAP-AR expression system can overcome this problem, but it is not without limitations. For example, the TAP module may destroy the native structure and related activity of the target protein or change its physical interaction(s) with native interaction partners inside cells. Unfortunately no affinity-based protein purification methodology is flawless. However, interfacing a large scale co-immunoprecipitation strategy with the ICAT method as reported here provides an alternative experimental approach for detecting protein complexes in mammalian cells. The reported AR-binding protein modules provide a molecular framework for developing and testing new hypotheses related to AR function in normal and neoplastic prostate epithelium. Future AR binding experiments will provide a more complete picture of AR interaction protein modules to better understand the multiple molecular and cellular functions of AR in mammalian cells. Experiments to identify AR protein complexes that nucleate on target androgen-responsive genes (e.g. PSA and FKBP5) are also underway as the molecular composition of these DNA-protein complexes has not been fully characterized. Lastly the reported putative AR-binding proteins may represent protein modules whose normal expression pattern becomes deregulated during AR-mediated prostate carcinogenesis in men.

**Summary**

We have developed a heterologous AR expression system in the 293HEK cell line that recapitulates AR SHR activity in PCa cells (2). This system identified putative AR-binding proteins in the cytosolic and nuclear extracts using the ICAT method (25, 35). Established AR-binding proteins (e.g. chaperones) that influence AR signaling in normal and neoplastic prostate epithelium were detected. More importantly, several groups of functionally related proteins were specifically enriched during AR purification (e.g. cytoskeletal, RNA binding, protein transport), suggesting a functional link between these protein networks and AR in vivo. AR binds to a large, functionally diverse group of proteins, making it difficult to fully understand the multiple activities of AR during the growth and differentiation of normal and neoplastic prostate epithelial cells in vivo. This study represents the first attempt at constructing a large scale AR-protein interaction map in mammalian cells using quantitative mass spectrometry. Interestingly the putative AR-binding proteins detected in this study showed little overlap to AR-binding proteins annotated at the McGill and HPRD databases. Many studies detected AR-binding proteins using truncated AR domains in the Y2H or GST-protein interaction assays (e.g. ligand-binding domain and DNA-binding domain) (18). The multidomain structure of AR makes it difficult to study protein-protein interactions to intact AR by these methods. The NTAP-AR expression system can overcome this problem, but it is not without limitations. For example, the TAP module may destroy the native structure and related activity of the target protein or change its physical interaction(s) with native interaction partners inside cells. Unfortunately no affinity-based protein purification methodology is flawless. However, interfacing a large scale co-immunoprecipitation strategy with the ICAT method as reported here provides an alternative experimental approach for detecting protein complexes in mammalian cells. The reported AR-binding protein modules provide a molecular framework for developing and testing new hypotheses related to AR function in normal and neoplastic prostate epithelium. Future AR binding experiments will provide a more complete picture of AR interaction protein modules to better understand the multiple molecular and cellular functions of AR in mammalian cells. Experiments to identify AR protein complexes that nucleate on target androgen-responsive genes (e.g. PSA and FKBP5) are also underway as the molecular composition of these DNA-protein complexes has not been fully characterized. Lastly the reported putative AR-binding proteins may represent protein modules whose normal expression pattern becomes deregulated during AR-mediated prostate carcinogenesis in men.
Androgen Receptor Protein Modules

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