Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system

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The AR (androgen receptor) is a ligand-regulated transcription factor, which belongs to the steroid receptor family and plays an essential role in growth and development of the prostate. Transcriptional activity of steroid receptors is modulated by interaction with co-regulator proteins and yeast two-hybrid analysis is commonly used to identify these steroid receptor-interacting proteins. However, a limitation of conventional two-hybrid systems for detecting AR protein partners has been that they only allow for analysis of the ligand- and DNA-binding domains of the receptor, as its NTD (N-terminal domain) possesses intrinsic transactivation activity. To identify AR N-terminus-interacting proteins, its NTD was used in the RTA (repressed transactivator) system, which is specifically designed for transactivator bait proteins and was shown to be suitable for two-hybrid analysis with the AR NTD. DDC (L-dopa decarboxylase) was detected multiple times as a novel AR-interacting protein, which was subsequently confirmed *in vitro* and *in vivo*. Furthermore, transient transfection of DDC in prostate cancer cells strongly enhanced ligand-dependent AR transcriptional activity, an effect that was antagonized using high concentrations of the anti-androgen bicalutamide. Glucocorticoid receptor activity was also strongly enhanced with DDC co-transfection, while oestrogen receptor activity was only mildly affected. Together, our data demonstrate that DDC interacts with AR to enhance steroid receptor transactivation, which may have important implications in prostate cancer progression.

Key words: androgen receptor, co-activator, prostate cancer.

INTRODUCTION

The AR (androgen receptor) is a ligand-activated transcription factor that belongs to the nuclear steroid hormone receptor family [1]. These receptors have similar functional domain structures that are composed of an NTD (N-terminal domain), a DBD (DNA-binding domain), a hinge region and an LBD (ligand-binding domain). In the absence of androgen, AR remains in the cytoplasm as an inactive complex, which includes Hsps (heat-shock proteins). The LBD of AR, in addition to forming the ligand-binding pocket, mediates the interaction between AR and these Hsps [2] and also interacts with the AR N-terminus to stabilize bound androgen [3]. After AR binds to its ligand, the receptor–ligand complex translocates to the nucleus, where it can bind to specific androgen-response elements found in the promoter of androgen-regulated genes [4]. Similar to other members of the steroid receptor family, AR contains two transcriptional AFs (activation functions); a constitutively active AF-1 located in the NTD of the receptor and a ligand-dependent AF-2 within the LBD [5].

The underlying mechanism through which AR specifically and differentially regulates gene expression remains uncertain. The DBD of AR is highly conserved among steroid receptors and recognizes the same steroid-response element consensus sequence as the GR (glucocorticoid receptor) [6]. Although subtle differences in the response element may dictate steroid receptor specificity, more recent evidence suggests that AR-specific gene regulation may also occur through interactions with unique co-regulatory proteins. These proteins can enhance (co-activators) or inhibit (co-repressors) the transcriptional activity of steroid receptors (reviewed in [7]). A number of co-regulators have been shown to interact with AR. These include SRC1 (steroid receptor co-activator 1) [8] and CBP [CREB (cAMP-response element-binding protein)-binding protein]/p300 [9], which facilitate the access of steroid receptors and basal transcriptional machinery to their target DNA sequences through chromatin remodelling and histone acetyltransferase activity. In addition, ARA70 (androgen receptor-associated protein 70 kDa) [10], cyclin D1 [11], caveolin [12], β-catenin [13] and several other proteins have also been found to interact with AR. Most of these co-regulator proteins have been isolated as a consequence of their binding to the DBD and LBD of steroid receptors. These domains share a high amount of sequence homology, and hence the proteins associated with these regions have been found to be indiscriminate in their ability to interact with and affect steroid receptor activity. Since the NTD of steroid receptors is the least conserved, protein interactions in this region may dictate receptor-specific co-regulation capacity. Indeed the GR N-terminal co-activator DRIP150 (vitamin D receptor-interacting protein 150 kDa) does not interact with the N-termini of other steroid receptors, including AR [14]. There are also several known AR NTD protein partners, which include the classical co-activators SRC1 [8] and CBP [9], the novel co-activator termed ART-27 (androgen receptor trapped clone

Abbreviations used: AR, androgen receptor; RTA, repressed transactivator; DDC, L-dopa decarboxylase; GR, glucocorticoid receptor; ERα, oestrogen receptor α isoform; NE, neuroendocrine; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; Hsp, heat-shock protein; AF, activation function; STAT3, signal transducers and activators of transcription 3; PLP, pyridoxal phosphate; DA, dopamine; 5-FOA, 5-flouro-orotic acid; GST, glutathione S-transferase; NP-40, Nonidet P-40; FBS, fetal bovine serum; E_2, 17β-oestradiol; SRC1, steroid receptor co-activator 1; CBP, cAMP-response element-binding protein-binding protein; ARA70, androgen receptor-associated protein 70 kDa; ART-27, androgen receptor trapped clone 27.

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27) [15], the transcription factor STAT3 (signal transducers and activators of transcription 3) [16] as well as the general transcription factor TFIIF [17]. However, the interaction of AR with SRC1 and CBP can occur through both the NTD and LBD of the receptor and is not unique, as these co-activators are known to interact with multiple nuclear receptors. In contrast, ART-27 has been shown to interact exclusively with the NTD of AR in yeast but also binds the N-terminus of GR and the ERα (oestrogen receptor α isoform). Furthermore, studies on the interaction of STAT3 and TFIIF with AR have been confined to the AR NTD, still allowing the possibility that they may also interact with the LBD of the receptor. Hence it remains to be seen whether there are co-regulator proteins that interact strictly with the unique amino acid sequence of the AR NTD and specifically modify AR transcriptional activity. Although the list of proteins proposed to bind to the AR N-terminus is expanding, it is likely that additional AR-binding partners remain to be found.

To identify novel AR-interacting proteins, its NTD was used as bait for yeast two-hybrid analysis. However, a major limitation of the AR NTD is that it contains intrinsic transactivation activity and therefore cannot be utilized as a bait for conventional yeast two-hybrid screening. To circumvent this problem, the RTA (repressed transactivator) system [18], specifically designed for transactivator bait proteins, was used to screen a prostate carcinoma cell line cDNA library for AR NTD protein partners. One of the clones detected multiple times in this screen coded for a partial sequence of DDC (L-dopa decarboxylase), also referred to as aromatic L-amino acid decarboxylase. Human neuronal DDC was originally cloned from a pheochromocytoma cDNA library and encodes a $\approx$50-kDa enzyme, as estimated by SDS/PAGE, with a single PLP (pyridoxal phosphate) cofactor-binding site [19]. The non-neuronal cDNA has also been cloned from human liver tissue. The non-neuronal and neuronal DDC mRNAs differ only in the region corresponding to the untranslated first exon and hence give rise to a single protein isoform [20]. DDC is responsible for decarboxylating both L-dopa and L-5-hydroxytryptophan into DA (dopamine) and 5-hydroxytryptamine ('serotonin'), respectively (reviewed in [21]). The distribution of DDC has been investigated in various brain regions as well as several peripheral tissues. It is widely distributed in neural tissues, where it plays a neuron-specific role as a neurotransmitter biosynthetic enzyme and in extra-neuronal tissues (adrenals, kidney, liver, gastrointestinal tract and lungs) where it acts as non-specific decarboxylating enzyme or may have other so-far-undetermined functions (reviewed in [22]). Furthermore, DDC has recently been suggested to be a biomarker for human prostate adenocarcinoma with NE (neuroendocrine) differentiation; a phenotype that has been associated with a more aggressive form of prostate cancer [23].

Here we demonstrate a novel role for DDC as an AR-binding protein. DDC was shown to interact with AR *in vitro* and *in vivo*, as well as to increase the transcriptional activity of the receptor in prostate cancer cell lines. This enhancement was inhibited when the transactivation assays were performed in the presence of bicalutamide (anti-androgen). Taken together, these results suggest that DDC may play an important role in AR gene regulation during prostate cancer progression.

MATERIALS AND METHODS

Plasmids and yeast strain

Three different regions of the human AR (AR_{1-559}, $AR_{233-559}$ and AR_{1-646}) were cloned downstream of the GAL4 DBD in pGBT9 (Clontech) to produce fusion proteins comprising GAL4 DBD and AR regions [24]. The vector used for construction of the LNCaP (a prostate cancer cell line) cDNA library, pGADT7-TUP1, was modified from pGADT7 (Clontech) in which the GAL4 activation domain was replaced with the N-terminal 200 residues of TUP1 (a general yeast transcriptional repressor protein), without disrupting the nuclear localization sequence, haemagglutinin epitope tag and T7 RNA polymerase promoter. The first step involved PCR amplification of the nuclear localization sequence containing a 5′ *Hin*dIII site (in italics) using primers (5′-ACTCC*AAGCTT*TGCAAAGATGGATAAA-3′ and 5′-GCGGCGGTACCCAATTCGACCTT-3′) on template pGADT7. This fragment was ligated to the PCR product of TUP1, amplified using primers that provide a *Bgl*II site (in italics) at the 3′ end (5′-ATGACTGCCAGCGTTTCGAATACG-3′ and 5′-ATTAA*AGATCT*CTGCCACGGAAACCTGGGGAGGTGG-3′) on template pBD2 [18]. The ligated DNA was then cloned into *Hin*dIII and *Bgl*II sites of pGADT7. The expression of TUP1 was confirmed using Western blotting, probed with an antibody to the haemagglutinin epitope. The yeast strain used was MAV103 (*MAT*α, *gal4*, *gal80*, *leu2*, *trp1*, *his3*, *SPAL10*::*URA3*) [25].

The full-length DDC expression vector, pDEST12.2-DDC, was constructed by reverse transcriptase-PCR on LNCaP RNA and Gateway™ cloning (Gibco-BRL). Primers used (5′-GGGGAC-AAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGA-ACC**ATGAACGCAAGTGAATTCCGAAGG**-3′ and 5′-GGG-GACCACTTTGTACAAGAAAGCTGGGTCCTA**CTCCCTC-TCTGCTCGCAGCAC**-3′) contained Shine–Dalgarno, Kozak and DDC gene-specific (in bold) sequences, along with the *att*B recombination sites allowing incorporation of the PCR product into pDONR201 to generate the pENTR-DDC entry vector. After frame and sequence verification, the entry vector was used in a second recombination site reaction with pDEST12.2 [which contains the CMV (cytomegalovirus) promoter for mammalian expression and SP6 promoter for *in vitro* transcription/translation] to produce the pDEST12.2-DDC expression plasmid.

Only the coding region of DDC was cloned, excluding any differences due to neuronal versus non-neuronal mRNA.

The pRC/CMV hAR_{1-646} expression plasmid was generated as described previously [24]. The full-length rat AR was also expressed from the pRC/CMV mammalian expression vector, pAR6 [26]. The rat GR (pGR) and human ERα (pERα) mammalian expression vectors have been described previously [27,28].

Construction of the TUP1–LNCaP cDNA fusion library

Total RNA was extracted from the LNCaP prostate cancer cell line using TRIzol according to the manufacturer's protocol (Gibco-BRL). Poly(A)$^+$ RNA was purified three times over oligo(dT)–cellulose columns (Boehringer Mannheim), and cDNA with *Eco*RI and *Xho*I ends was synthesized using the Stratagene cDNA Synthesis Kit, which was ligated into appropriately digested pGADT7 vector with the TUP1 sequence. Ligated DNA was electroporated into ElectroMAX DH10B cells (Gibco-BRL). The cDNA plasmid library was amplified using the semi-solid amplification method (Stratagene) and DNA was purified over a $CsCl_2$ gradient [29]. The library was estimated to contain 6×10^6 independent clones, and about 81 % of the clones contained an insert.

GAL4-hAR NTD RTA screen

Yeast strain MAV103 was co-transformed with one of the three pGBT9-hAR baits (AR_{1-559}, $AR_{233-559}$ and AR_{1-646}) and the TUP1–LNCaP cDNA library plasmid. Cells were plated on minimal medium (− Leu, − Trp) in the presence of 5-FOA

(5-flouro-orotic acid). The concentration of 5-FOA used was determined by co-transforming yeast with bait and either TUP1–LNCaP cDNA library or empty pGADT7-TUP1 vector and plating on media with increasing 5-FOA concentration (0.01–0.1 % at 0.005 % intervals). The minimum amount of 5-FOA that caused 100 % of the cells to die was chosen for screening the library. Colonies that survived selection were collected on days 4, 5, 6 and 7 and were passaged once on to fresh 5-FOA plates. Isolated colonies were expanded in 200 μl of minimal medium and DNA was extracted using the Yeast DNA Extraction Reagent (Y-DER) Kit (Pierce); cDNA inserts were amplified by PCR using a TUP1 internal primer and a vector-specific primer. PCR products were purified over MinElute columns (Qiagen) and sequenced using the Big Dye Terminator Sequencing Kit (ABI Prism, PE Applied Biosystems). The sequence was compared with known sequences available from GenBank™. Positive clones that had appeared multiple times were passaged into HB101 bacteria (which carry a selectable marker for leucine) for cDNA plasmid isolation. Positive interactions with the bait were re-confirmed in yeast.

GST (glutathione S-transferase) pull-down assays

Various fragments of the rat AR ($AR_{234-665}$, $AR_{541-665}$ and $AR_{541-919}$) and an N-terminal fragment of the human AR lacking the DBD (AR_{1-559}) were cloned into the pGEX vector for expression of GST-fusion proteins in *Escherichia coli* BL21 cells, as described previously [30]. For semi-quantitative analysis of binding affinity between regions of DDC and AR, the volume of resuspended GST–AR-domain fusion protein-bound beads corresponding to an equimolar amount of each fusion protein was determined initially. Fusion protein-bound bead volumes were titrated, eluted with sample buffer (2 % SDS and 5 % β-mercaptoethanol) and analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining. The eluent in each case was run alongside known amounts of BSA (ranging from 250 to 1000 ng) to generate a standard curve for protein concentrations. Band intensities were measured (Gel Doc 2000, Quantity One; Bio-Rad) and the amount of GST-fusion protein corresponding to specific bead volumes (fusion protein bound) was calculated from the BSA standard curve (results not shown). The estimated volume of fusion protein bound-beads containing the proper amount of protein was then re-analysed using SDS/PAGE, as above. This was done in triplicate to ensure that bead volumes used in the pull-down assays contained an equimolar concentration of each GST–AR domain fusion protein.

The pDEST12.2-DDC vector was used to prepare ^{35}S-radiolabelled full-length DDC with the Quick Coupled SP6 TnT *in vitro* transcription/translation kit (Promega). The same vector was also used for *Eco*57I digestion (which has a single cut site at nucleotide 1071 of DDC) and preparation of the radiolabelled DDC N-terminal fragment (DDC_{1-333}). The C-terminal DDC fragment ($DDC_{328-480}$) was translated (T7 promoter kit) from the pGADT7-$DDC_{328-480}$ yeast two-hybrid clone vector.

Equimolar amounts of GST–AR fusion protein coupled to glutathione beads were incubated with radiolabelled full-length or fragments of DDC at 4 °C for 2 h in binding buffer [20 mM Hepes, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA and 0.05 % NP-40 (Nonidet P-40)]. Beads were washed four times with binding buffer and bound proteins were eluted in sample buffer for SDS/PAGE and autoradiographic visualization. Dried gels were also analysed using a Phosphorimaging screen and the Molecular Imager FX (Bio-Rad). Quantity One software was used to obtain [CNT*mm^2] data (counts/mm^2) for radiolabelled protein bands. All pull-downs were done in triplicate and averaged [CNT*mm^2] was normalized as a function of the percentage input bound.

Mammalian cell culture and transfection

Human prostate cancer PC3 and DU145 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5 % FBS (fetal bovine serum; Gibco-BRL) at 37 °C in 5 % CO_2. LNCaP prostate carcinoma cells were cultured in RPMI media containing 5 % FBS. Transactivation assay transient transfections were carried out in six-well plates. Cells were seeded at a density of 3×10^5 cells/well and transfected the following day using Lipofectin Reagent (Gibco-BRL) as described previously [31]. Cells were incubated with transfection mix for 16 h at 37 °C and subsequently re-fed with 5 % dextran-coated charcoal-stripped FBS containing 1 nM R1881, 10 nM dexamethasone, 10 nM E_2 (17β-oestradiol) or vehicle alone. For anti-androgen studies cells were re-fed with 1 nM R1881 and varying concentrations of bicalutamide (provided by Astra-Zenaca, Wilmington, DE, U.S.A.) or vehicle alone. The cells were then incubated for 24 h at 37 °C before lysis in passive lysis buffer (Promega) for luciferase assay and Western blot analysis.

Co-immunoprecipitation and Western blotting

LNCaP cells (2×10^6) were plated on 10-cm dishes and grown to 70 % confluency in RPMI 1640 with 5 % FBS. Cells were then transfected with pDEST12.2-DDC vector (5 μg/dish) using Lipofectin Reagent. Medium was changed to RPMI 1640 containing 5 % dextran-coated charcoal-stripped FBS and incubated for a further 16 h at 37 °C to deplete cells of bio-available hormone. Cells were then treated with or without 10 nM R1881 for 4 h before harvest. Washed cells were resuspended in 0.5 % NP-40 buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 2 mM EDTA and 0.5 % NP-40) for lysis. Cell extracts [1 mg of protein, as quantified by BCA (bicinchoninic acid) assay] were incubated with a monoclonal mouse anti-AR_{DBD} antibody (BD Biosciences, PharMingen) or with an equivalent amount of normal mouse IgG (negative control). Recombinant Protein G–agarose (Invitrogen) beads were used to immunoprecipitate antibody–protein complexes. Beads were washed four times with lysis buffer and resuspended in $2 \times$ SDS sample buffer. Associated proteins were resolved by SDS/PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) as described previously [26]. Membranes were blocked in 5 % skimmed milk in Tris-buffered saline (20 mM Tris/HCl, pH 7.6/140 mM NaCl) prior to incubation with the appropriately diluted primary antibody. AR and DDC were detected using rabbit polyclonal antibodies (Affinity BioReagents and Chemicon, respectively). Blots were developed using horseradish peroxidase-conjugated secondary antibody and the ECL chemiluminescence kit (Amersham Biosciences). To verify the specificity of the anti-$DDC_{N\text{-terminal}}$ antibody (Chemicon), a competition assay was carried out using Ni^{2+}-nitrilotriacetate (Qiagen) affinity-purified $6 \times$ His-tagged DDC protein. This purified protein was incubated with primary antibody solution before performing the Western blot (10:1 molar ratio of $6 \times$ His-DDC to antibody). Antibodies for GR and ERα were obtained from Transduction Laboratories (BD Biosciences) and Santa Cruz Biotechnology, respectively.

Transcriptional assays

The pARR3-tk-Luc reporter construct [30], which has three tandem ARRs (androgen-response regions), was used for assaying AR and GR transcriptional activity. ERα activity was monitored

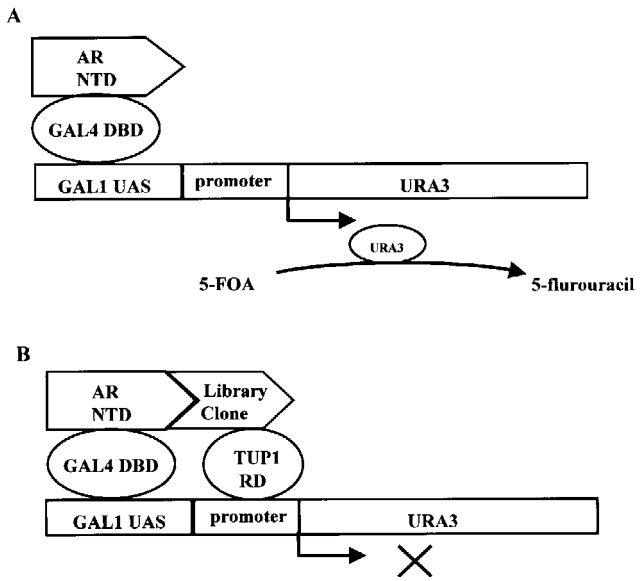
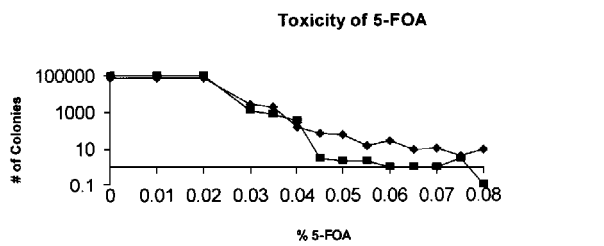


Figure 1 AR NTD as bait in repressed transactivator two-hybrid system

The yeast strain used was MAV103, which contains multiple GAL4-binding sites (UAS) upstream of a *URA3* reporter gene. The RD (repression domain) of TUP1 is fused to the LNCaP cDNA library. (**A**) Activation of the GAL1-URA3 reporter gene by GAL4 DBD–AR NTD fusion activator bait protein causes constitutive expression of orotidine-5′-phosphate decarboxylase, leading to 5-FOA sensitivity. (**B**) Interaction of a TUP1–LNCaP cDNA library fusion prey and the NTD activator bait causes transcriptional repression of the *URA3* reporter, resulting in 5-FOA resistance.



B

1 mnasefrrrg kemvdyvany megiegrqvy pdvepgylrp lipaaapqep dtfediindv

61 ekiimpgvth whspyffayf ptassypaml admlcgaigc igfswaaspa cteletvmmd

121 wlgkmlelpk aflnekageg ggviqgsase at**lvall**aar tkvihrlqaa speltqaaim

181 eklvayssdq ahssveragl iggvklkaip sdgnfamras alqealerdk aaglipffmv

241 atlgtttccs fdnllevgpi cnkediwlhv daayagsafi cpefrhllng vefadsf*nfn*

301 *phkw*llvnfd csamwvkkrt dltgafrldp tylkhshqds glitdyrhwq iplgrrfrsl

361 kmwfvfrmyg vkglqayirk hvqlshefes lvrqdprfei cvevilglvc frlkgsnkvn

421 eallqrinsa kkihlvpchl rdkfvlrfai csrtvesahv qrawehikel aadvlraer*

Figure 2 Detection of DDC as an AR NTD-interacting protein

(**A**) Concentration of 5-FOA used for screening was determined by co-transformation of yeast with bait (GAL4 DBD–AR_{1-559}) and TUP1–LNCaP cDNA library (◆) or empty pGADT7-TUP1 vector (■) before plating on media with increasing 5-FOA concentration. (**B**) The amino acid sequence of DDC (GenBank™ accession number NM 000790) is shown, with an asterisk representing the stop codon. Six independent clones coding for DDC were detected. The underlined region shows the protein encoded by one of the cDNA sequences isolated. Italicized residues represent the PLP cofactor-binding site of DDC and emboldened residues show the single LXXLL motif.

with the ERE-Luc reporter, which contains a single vitellogenin oestrogen-response element upstream of a thymidine kinase promoter. PC3 and LNCaP cells, transfected in six-well plates, had total DNA amounts adjusted to 3 μg/well using pRC/CMV vector. Cells were treated with or without hormone for 24 h at 37 °C prior to analysis. The *Renilla* luciferase vector, pRL-TK (Promega), was used for normalization of transfection efficiency. The Dual Luciferase Assay kit (Promega) and MicroLumiat*Plus* luminometer (EG&G Berthold) were used to assay firefly and *Renilla* luciferase activities. All assays were done in triplicate, with at least three independent trials in each case.

RESULTS

AR N-terminus is a suitable bait for the RTA system

To identify proteins that bind to the AR NTD, we screened a LNCaP cDNA library for interacting proteins using a yeast two-hybrid system. The N-terminus of AR has been shown to contain strong intrinsic transactivation activity in yeast when fused to the GAL4 DBD. For this reason, we used a reverse yeast two-hybrid screen that employs the RTA system specifically designed for transactivator bait proteins [18]. As with the conventional yeast two-hybrid system, the AR NTD is fused to a GAL4 DBD to form the bait. However, the prey is created by fusion of the cDNA library with a TUP1 repressor protein rather than with an activation domain. Since the N-terminus of TUP1 is sufficient to cause transcriptional repression, only the first 200 amino acid residues were fused to the cDNA library [18,32]. When there is no interaction between the bait and TUP1-library fusion protein, the bait activator allows constitutive expression of the *URA3* reporter gene (Figure 1A). This permits growth in the absence of uracil, but causes sensitivity to 5-FOA, which is converted into a toxic 5-flourouracil product through the enzyme encoded by the *URA3* gene. Thus transcriptional repression can be genetically monitored by using a counter-selectable *URA3* reporter [33]. In a situation where there is an interaction between the AR NTD and a library clone, TUP1 will cause repression of URA3 expression, which can be detected by growth of yeast in the presence of 5-FOA (Figure 1B). Therefore, viability of the MAV103 strain, or colony growth, on 5-FOA media represents a positive interaction between the AR NTD bait and prey.

Detection of DDC as an AR N-terminus-interacting protein in yeast

The RTA system has not been previously used to identify novel AR-interacting proteins. We examined three overlapping regions of the human AR (amino acids 1–559, 233–559 and 1–646) as separate baits in this system. All three baits contain the AR N-terminal region (amino acids 360–494) that is responsible for the intrinsic transactivation activity of AR when its LBD is deleted [34]. Initial toxicity tests were done to generate dose–response curves to 5-FOA and determine the optimal concentration for screening. The minimum amount of 5-FOA that caused $\approx$100 % cell death in the absence of the library, and a significant increase in colony survival with the library, was chosen as the optimal concentration for screening with that particular bait (increased by an increment of 0.01 % to ensure maximal stringency). Optimal 5-FOA concentration for screening with the AR full N-terminus (AR_{1-559}) bait was determined to be 0.055 % (Figure 2A). Similarly, concentrations of 5-FOA used for the $AR_{233-559}$ and AR_{1-646} baits were found to be 0.06 and 0.055 %, respectively (results not shown).

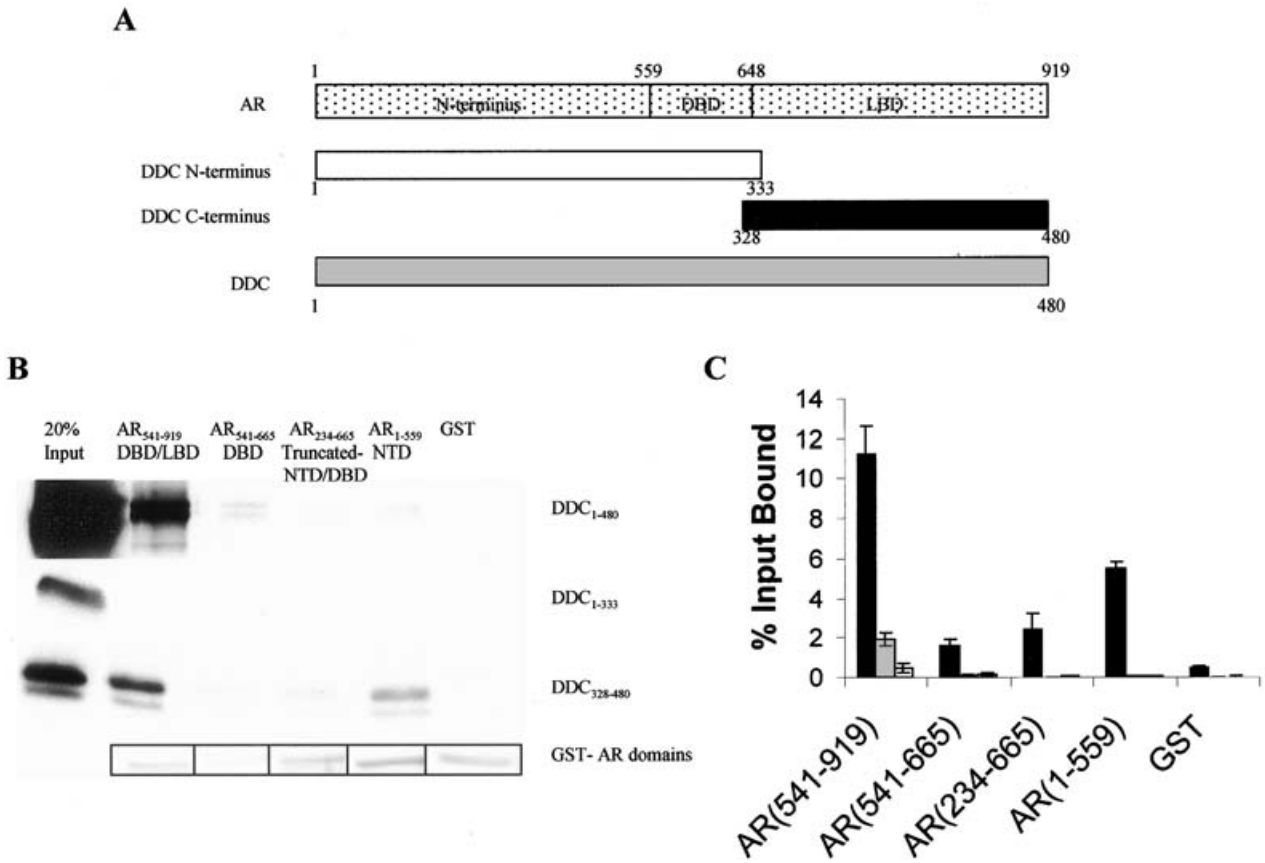


Figure 3 DDC interacts with AR *in vitro* through its C-terminus (328–480)

(**A**) Diagram of AR functional domains and DDC regions used in GST pull-down assays (open, closed and stippled regions correspond to bars in panel **C**). (**B**) GST protein and four GST–AR domain fusion proteins ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$ and AR_{1-559}) were expressed in bacteria and coupled to glutathione–agarose beads at equimolar levels, as determined by Coomassie Brilliant Blue stain analysis and a BSA standard curve. Full-length DDC and fragments (DDC_{1-333} and $DDC_{328-480}$) were expressed by *in vitro* translation and radiolabelled using [^{35}S]methionine. The DDC_{1-333} fragment was generated by restriction digest of the pDEST12.2-DDC vector with *Eco*57I before labelling. After incubation of radiolabelled proteins and GST–AR domains, bound protein was eluted for SDS/PAGE and autoradiography analysis. (**C**) Phosphorimager and Quantity One software were used to quantify bound protein from the loaded input. All pull-downs were done in triplicate and averaged [CNT*mm^2] was used to assess binding strength quantitatively as a function of the percentage of input bound.

Approx. 3×10^6 transformants were screened with all three AR NTD baits and the positive clones were sequenced and compared with known sequences available in GenBank™. A total of 40 unique in-frame positive clones were detected from the LNCaP cDNA library, some of which were picked up multiple times. These included: a steroid receptor chaperone Hsp (only detected with the DBD-containing AR_{1-646} bait); the cyclin G-associated Ser/Thr kinase [35], potentially involved in AR phosphorylation; a subunit belonging to one of the general transcription factors; a transcriptional adaptor protein and several previously undescribed AR protein partners (all currently under investigation). The most frequently detected clone coded for the C-terminal region of DDC (or aromatic L-amino acid decarboxylase; EC 4.1.1.28, GenBank™ accession number NM 000790) [19]. DDC was detected a total of six times, with all three AR NTD baits. DDC is a well-characterized enzyme responsible for production of DA and 5-hydroxytryptamine, but has been suggested to have other unknown functions in peripheral tissues (reviewed in [21,22]). The six library clones isolated with the RTA yeast two-hybrid assay were all ≈900 bp, one of which coded for amino acids 328–480 of DDC (pGADT7-$DDC_{328-480}$) and the 3′-untranslated region (Figure 2B). The full-length DDC coding region was then cloned into the Gateway™ pDEST12.2 mammalian expression vector (pDEST12.2-DDC) using reverse transcriptase-PCR on LNCaP RNA.

DDC interacts with LBD and NTD of AR through its C-terminus

To confirm results of the two-hybrid screen, GST pull-down assays were performed using a series of GST-fused AR fragments (Figure 3). For quantitative assessment of binding affinity between various domains of AR and radiolabelled DDC, initial equimolar normalization of GST–AR-domain fusion proteins was carried out, as described in detail the Materials and methods section. Pull-down assays were performed in triplicate and the averaged [CNT*mm^2] was used to calculate percentage of total input bound ([CNT*mm^2] bound/[CNT*mm^2] input) to the GST–AR domains. With this type of analysis, differences in binding between AR and DDC can be attributed to actual affinity rather than differential expression or binding ability of the GST–AR-domain fusion proteins to the glutathione beads. Figure 3(B) shows the Coomassie Blue stain used for normalization of the GST–AR domains ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$ and AR_{1-559}), as well as the autoradiogram of one representative GST pull-down assay with radiolabelled DDC. Full-length DDC protein does interact with the AR_{1-559} (NTD) fragment, which was one of

three baits used in the RTA yeast two-hybrid screen. However, this interaction is much weaker than that of the $AR_{541-919}$ (DBD/LBD) fragment. Since the $AR_{541-665}$ (DBD) interaction is also weak, the strong binding of full-length DDC protein to AR can be largely attributed to its affinity for the LBD of AR. Overall, full-length DDC interacts with all domains of AR, with the strongest interaction occurring at the AR LBD.

To determine the region of DDC responsible for interaction with AR, N-terminal (DDC_{1-333}) and C-terminal ($DDC_{328-480}$) fragments of the protein were radiolabelled and used for GST–AR domain pull-down analysis (Figure 3B). A summary of the quantified GST pull-down data (percentage of the input bound) for these DDC truncations and the full-length protein is shown in Figure 3(C). Similar to the full-length protein, $DDC_{328-480}$ interacts most strongly with the LBD-containing fragment ($AR_{541-919}$) of AR, but has ≈5-times higher affinity for this region (11 % versus 2 % of total input bound). The difference in binding affinity for the AR_{1-559} NTD fragment is even more drastic. A negligible proportion of full-length DDC input binds to AR_{1-559}, as compared with the 5 % of input bound for $DDC_{328-480}$. This strong affinity of the DDC C-terminal region for the N-terminus of AR may explain its high incidence as a positive clone in the RTA screen, where all six detected DDC clones were incomplete C-terminal cDNAs of similar length. The N-terminal fragment of DDC (residues 1–333) does not interact with any domains of AR (Figures 3B and 3C) *in vitro*.

DDC is expressed in prostate cancer cell lines

A polyclonal antibody (Chemicon) raised against an N-terminal peptide of human DDC was used to determine protein expression in three different prostate cancer cell lines. Western blot analysis of LNCaP, PC3 and DU145 cell lysates show that this antibody recognizes a single endogenous protein at the expected molecular mass of ≈50 kDa in LNCaP cells (Figure 4A, lane 1), but in PC3 and DU145 cells a lower ≈35-kDa band is also detected (Figure 4A, lanes 2 and 3). The endogenous protein migrates at the same apparent molecular mass as ectopically expressed DDC (LNCaP cells transfected with pDEST12.2-DDC vector; Figure 4A, lane 4). An immuno-competition assay was carried out to determine specificity of the antibody using purified 6 × His-DDC protein. As can be seen from Figure 4(A), lanes 5–8, incubation of antibody solution with 6 × His-DDC protein, prior to immunoblotting, selectively prevents detection of the ≈50-kDa band for all cell lines. Thus the ≈35-kDa band seen in PC3 and DU145 cells is non-specific. This confirms that endogenous DDC protein is present in these prostate cancer cells. A β-actin antibody was used to check loading efficiency in all lanes.

DDC interacts with AR *in vivo*

The presence of DDC protein in LNCaP cells and the strong *in vitro* interaction of this protein with AR suggested the possibility that DDC may be able to interact with the receptor in the intact cell. Since LNCaP cells express high levels of AR and are androgen sensitive, this prostate carcinoma cell line was used to assess the association of DDC with AR *in vivo* in the presence and absence of androgen. Cell extracts from untreated or 10 nM R1881-treated LNCaP cells (transfected with pDEST12.2-DDC) were subjected to immunoprecipitation with an anti-AR_{DBD} antibody and analysed by Western blot with antibodies to the N-termini of DDC and AR. DDC co-immunoprecipitated with AR in the presence and absence of R1881 (compare lanes 4 and 6 in Figure 4B), but the association seems to be stronger with the androgen treatment.

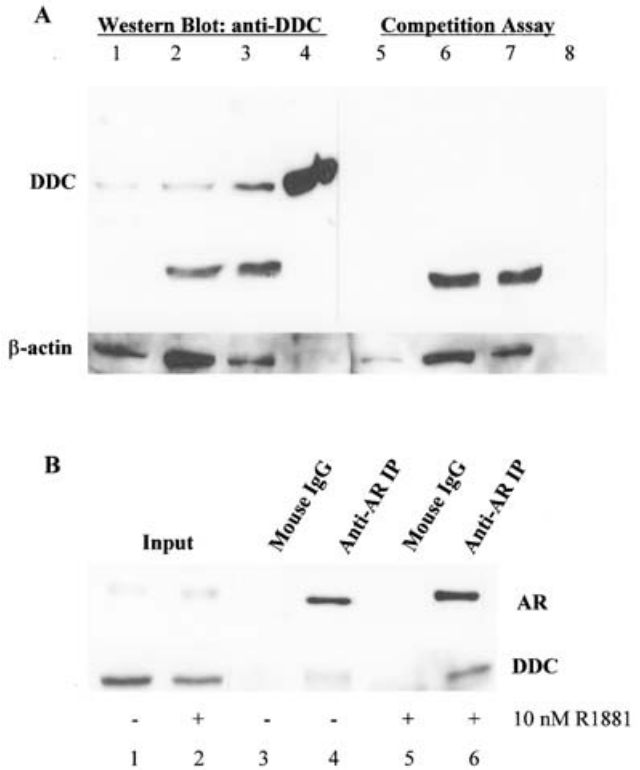


Figure 4 DDC is expressed in prostate cancer cell lines and co-immunoprecipitates with AR *in vivo*

(**A**) DDC protein expression was analysed in three prostate cancer cell lines. Equal amounts (50 μg) of cell extract (0.5 % NP-40 lysis) prepared from LNCaP (lanes 1 and 5), PC3 (lanes 2 and 6) and DU145 (lanes 3 and 7) cells were analysed by immunoblotting with an anti-$DDC_{N\text{-terminal}}$ antibody. A positive control (2 μg of cell extract from pDEST12.2-DDC-transfected LNCaP cells) was also included (lanes 4 and 8). Purified 6 × His-DDC protein was used for the immuno-competition assay (lanes 5–8). Protein loading efficiency was normalized by β-actin. (**B**) Co-immunoprecipitation; LNCaP cells were transfected with pDEST12.2-DDC and grown in the absence (lanes 1, 3, 4) or presence (lanes 2, 5, 6) of 10 nM R1881 for 4 h prior to lysis in 0.5 % NP-40 buffer. Cell extracts were incubated with an anti-AR_{DBD} antibody (lanes 4 and 6) or normal mouse IgG control (lanes 3 and 5). Input protein samples were loaded in lanes 1 and 2. Protein complexes were pulled-down using recombinant Protein G–agarose beads prior to SDS/PAGE and Western blot analysis, with antibodies raised against the N-termini of both DDC and AR.

DDC enhances AR transcriptional activity

To examine the biological impact of the DDC-AR interaction, transient transfection transactivation assays were carried out using PC3 cells. This prostate cancer cell line expresses little, if any, endogenous AR and hence levels of transfected AR to DDC can be titrated. PC3 cells were transfected with a constant amount of full-length AR vector (pAR_6) and increasing amounts of DDC expression vector (pDEST12.2-DDC), along with the pARR3-tk-Luc reporter plasmid. The ratio of AR:DDC was varied from 1:0, with no DDC transfected, to 1:10, where 10 times more DDC was transfected than AR. As can be seen in Figure 5(A), ligand-dependent AR activity was enhanced with increasing DDC expression; there was a maximum of ≈15-fold at the 1:10 co-transfection ratio. This enhancement of AR transactivation did not result from increased AR protein production, since receptor levels remained constant with DDC co-expression, as determined by Western blot analysis. The effect of DDC on AR activity was not restricted to a single cell type. Similar transient transfection assays in LNCaP cells also enhanced ligand-dependent AR

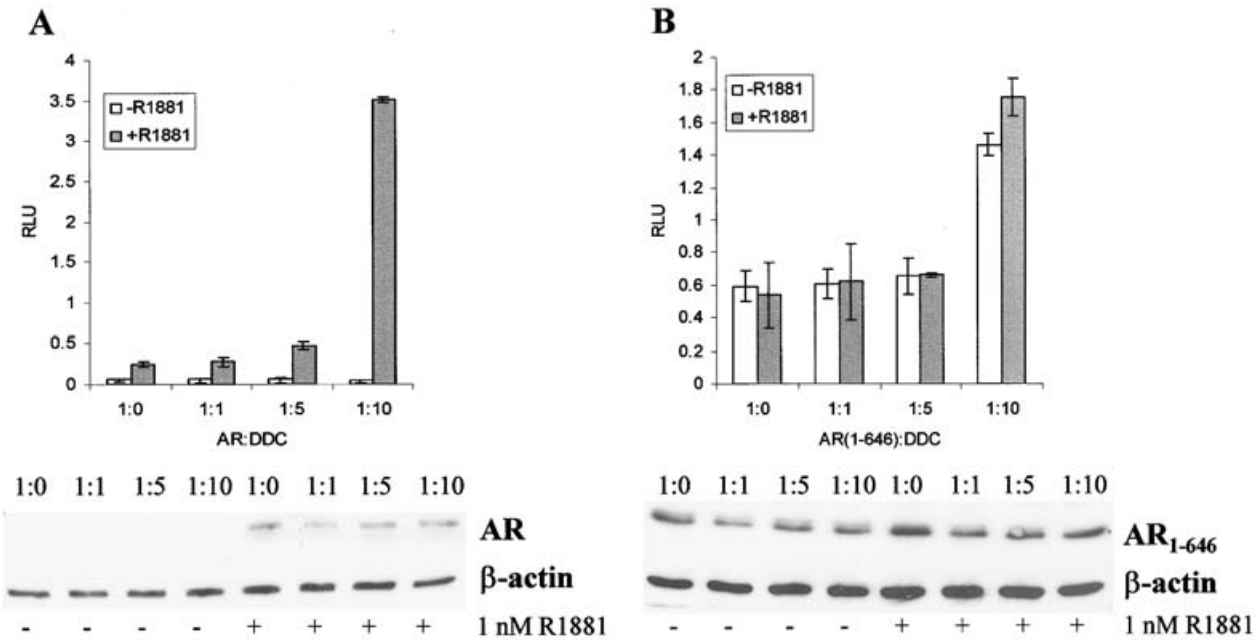


Figure 5 DDC enhances AR transcriptional activity in PC3 cells

(**A**) DDC strongly enhances AR activity. PC3 cells were transfected with full-length AR (250 ng/well), pARR3-tk-Luc (167 ng/well), pRL-TK-*Renilla* (83 ng/well) and increasing amounts of pDEST12.2-DDC (CMV promoter). These amounts were 0, 250, 1250 or 2500 ng/well and correspond to the 1:0, 1:1, 1:5 and 1:10 AR/DDC ratios, respectively. Total DNA was kept constant at 3 μg/well with addition of empty pRC/CMV vector. Cells were induced in the presence (closed bars) or absence (open bars) of 1 nM R1881 for 24 h before harvest and luciferase assay. (**B**) DDC increases ligand-independent activity of AR. PC3 cells were transfected and treated as above, but the LBD truncated AR_{1-646} (250 ng/well) was substituted for full-length AR. Transfection efficiency was normalized with the *Renilla* luciferase pRL-TK vector. RLU (relative light unit) values are the means $\pm$ S.D. from triplicate experiments. Each graph is representative of three independent trials. To determine receptor expression levels, 10 μg of protein lysate from each triplicate was combined (30 μg of total protein) and subjected to SDS/PAGE and Western blot analysis. Full-length AR and AR_{1-646} were detected using the anti-AR NTD antibody. Protein-loading efficiency was normalized using β-actin.

transcriptional activity 2–3-fold for the 1:10 ratio (results not shown). In the absence of ligand, AR activity was negligible and did not change with DDC co-transfection.

Since DDC was originally identified as an AR NTD-interacting protein in the yeast two-hybrid screen, the above transactivation assays were repeated using a LBD truncated form of AR (AR_{1-646}). As can be seen from Figure 5(B), AR_{1-646} is constitutively active in the presence and absence of androgen. DDC co-transfection (1:10 ratio) enhanced its activity about 2–3-fold. Western blot analysis was carried out to ensure that the truncated AR_{1-646} levels remained constant in all conditions. Overall, DDC seems to weakly enhance AR AF-1 activity and strongly enhance full-length AR transactivation, containing both AF-1 and AF-2 functions.

DDC enhances transcriptional activity of other steroid receptors

To determine whether the biological effect of DDC on AR is receptor-specific, the transient transfection assays were repeated in PC3 cells with GR and ERα (pGR and pERα). Figure 6(A) shows that DDC co-transfection increased GR ligand-dependent transcriptional activity up to 20-fold (1:10 ratio) in the presence of 10 nM dexamethasone. Similar to AR, GR activity remained unchanged in the absence of ligand. Therefore, DDC co-expression seems to enhance GR-mediated transcription in a manner similar to that of AR in PC3 cells. However, the effect of DDC on ERα activity in these cells seems to be much weaker. In the presence of 10 nM E_2 there is only about a 2-fold increase in ERα activity with high levels (1:10 ratio) of DDC expression (Figure 6B). Western blot analysis was used to monitor GR and ERα expression. Similar to AR, after normalization with the β-actin loading control, these receptor protein levels remained constant. The ability of DDC to enhance transactivation of other steroid receptors is not surprising since the full-length protein interacts strongly with the LBD of AR *in vitro*. This domain is well conserved among members of the steroid receptor family and may be the region through which DDC generally binds to enhance transcriptional activity.

Bicalutamide (anti-androgen) inhibits the enhanced AR transcriptional activity observed with DDC over-expression

The association of DDC with AR and the resultant enhancement of ligand-dependent AR activity, taken together with the recent report of DDC expression in advanced NE phenotype human prostate cancer [23], suggest that this protein may play an important role in disease progression. To investigate the possible role of DDC in anti-androgen refractory activation of AR in prostate cancer, the above transactivation assays were performed using PC3 (AR-transfected) and LNCaP (which express endogenous mutant AR) cells treated with or without the pure anti-androgen bicalutamide. As shown in Figure 7(A), in PC3 cells bicalutamide at a concentration of 10 μM could drastically reduce the AR transcriptional activity stimulated by 1 nM R1881, and at 50 μM bicalutamide AR activity was almost completely blocked. In contrast, DDC-transfected PC3 cells treated with 1 nM R1881 and 10 μM bicalutamide had a partial inhibition in AR activity and had lower, but still significant, activity at 50 μM bicalutamide. As expected [36,37], we were able to substantially block the activity of the mutant AR in LNCaP cells using

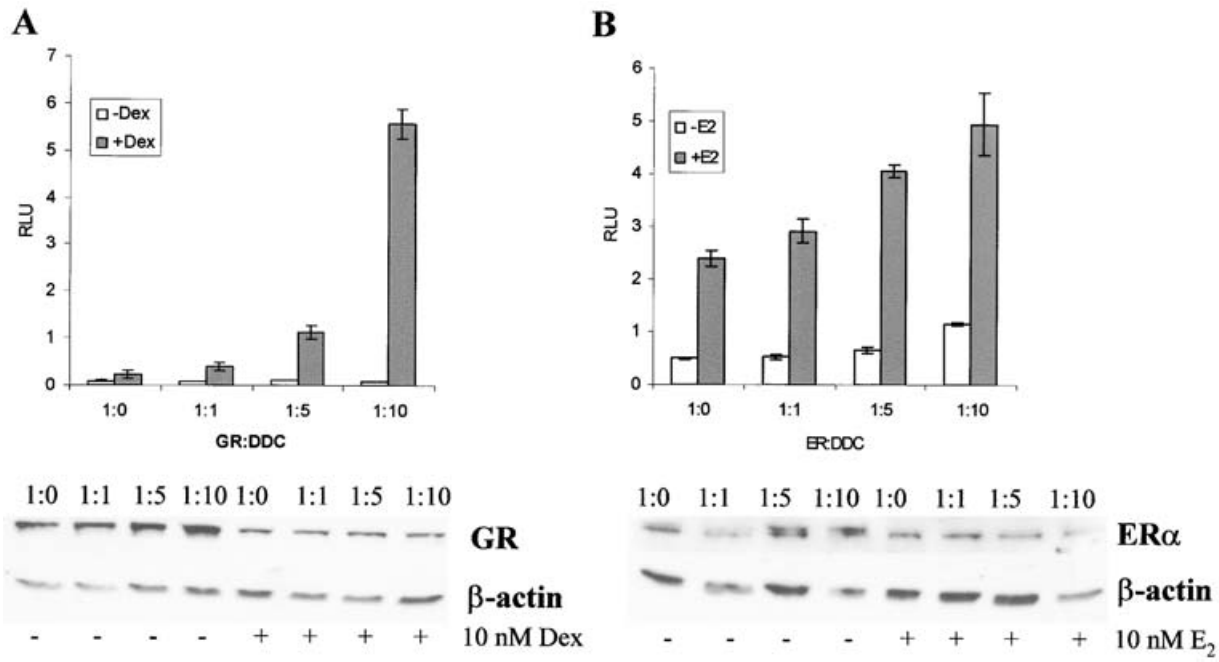


Figure 6 DDC strongly enhances GR transactivation and mildly increases ERα activity

PC3 cells were transfected with (**A**) GR (250 ng/well) or (**B**) ERα (250 ng/well) and the corresponding reporter (pARR3-tk-Luc and pERE-Luc, respectively). The ratio of steroid receptor to DDC was varied as outlined in Figure 5. Cells were treated with or without 10 nM dexamethasone (Dex; GR) or 10 nM E_2 (ERα) for 24 h prior to harvest and luciferase assay. Transfection efficiency was normalized with the *Renilla* luciferase pRL-TK vector. RLU (relative light unit) values are the means ± S.D. from triplicate experiments. Each graph is representative of three independent trials. SDS/PAGE and Western blot analysis were carried out as described in Figure 5 using anti-GR and anti-ERα antibodies.

bicalutamide (Figure 7B). As seen previously, the transfection of DDC augmented ligand-dependent AR transcriptional activity and similar to PC3 cells, the activity of AR was higher at both concentrations of bicalutamide when DDC was overexpressed. Nevertheless, the enhanced AR activity seen with DDC transfection was significantly reduced with bicalutamide in both PC3 and LNCaP cells, even though this required higher concentrations of the anti-androgen. These results suggest that DDC functions by augmenting the transcriptional activity of AR through a ligand-mediated mechanism.

DISCUSSION

In this study we have demonstrated that the RTA yeast two-hybrid system can be used as a novel screening method for detecting AR NTD-interacting proteins. Recent reports have shown use of modified conventional LexA yeast two-hybrid screens to identify AR NTD protein partners [15,38]. In these cases, the AR NTD is expressed as a galactose-inducible fusion protein linked to a B42 activation domain, while the cDNA library is fused to a LexA DBD. However, this screening method can result in a high false-positive detection rate from library clones that possess intrinsic transcriptional activation potential. Also, fusion of the cDNA library to a DBD, such as that of GAL4 or LexA, may alter its ability to bind the upstream activation sequence, introducing another level of variation in the two-hybrid assay. Alternatively, using the AR NTD as bait in the RTA system allows detection of clones that contain intrinsic transcriptional activity and does not require fusion of the cDNA library to a heterologous DBD. In our screen of a LNCaP cDNA library with AR NTD baits, we identified several previously undescribed interacting proteins, including a chaperone Hsp (detected with the DBD-containing AR_{1-646} bait), cyclin G-associated Ser/Thr kinase [35], a general transcription factor component and a transcriptional adaptor protein (all currently being analysed). These appear to be direct interactions, as protein produced by *in vitro* transcription/translation of the cDNA inserts did bind to the AR NTD in GST pull-down studies (results not shown). Surprisingly, in our screen, we did not identify known NTD-binding partners of AR, such as SRC1 [8], CBP [9], ART-27 [15], STAT3 [16] and TFIIF [17]. It is possible that these interactions are too weak to be identified at the 5-FOA concentration used or that fusion of these proteins to TUP1 may lead to toxicity in yeast. Furthermore, detection of these AR NTD-interacting proteins is dependent on the presence of the proper sequence in the cDNA inserts of the library, which are not complete for many clones.

Using the RTA system DDC was initially identified as a protein that interacts with the AR NTD in yeast. Surprisingly, *in vitro* GST pull-down analysis with various AR domains suggest that the main region of interaction between full-length DDC protein and AR occurs at the C-terminus (amino acids 328–480) of DDC and the LBD of AR (Figure 3). The initial detection of DDC as an AR NTD-interacting protein could be a consequence of the strong affinity that the truncated $DDC_{328-480}$ region has for the AR_{1-559} domain (Figure 3). Indeed, all six isolated DDC clones from the screen were incomplete C-terminal cDNAs of similar length. Therefore, use of the AR NTD as bait in the RTA screen can detect the library clones that interact strongly with the AR N-terminus. Coincidentally, the same DDC C-terminal region was found to interact twice as strongly with the LBD of AR. The dual interaction of $DDC_{328-480}$ with the LBD and NTD of AR was not surprising, since several other AR protein partners also interact with the receptor in a similar manner [8,9]. Compared with the $DDC_{328-480}$ fragment, the full-length protein has a lower binding affinity for all AR domains and exhibits almost a complete loss

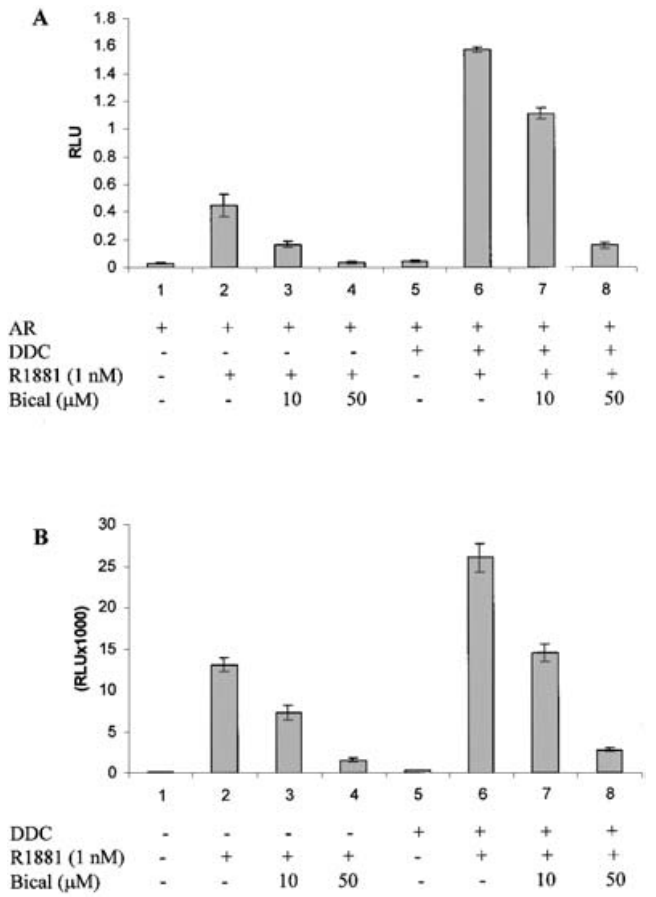


Figure 7 Effect of DDC on transactivation activity of AR in the presence of bicalutamide in prostate cancer cells

(**A**) PC3 cells were co-transfected with full-length AR (250 ng/well), pARR3-tk-Luc (167 ng/well), pRL-TK-*Renilla* (83 ng/well) and either 1 μg of pDEST12.2-DDC (DDC) or empty vector. After transfection, cells were treated with 1 nM R1881 and bicalutamide (Bical) or 1 nM R1881 and vehicle alone for 24 h, before harvest and luciferase assay. (**B**) LNCaP cells were co-transfected with the same plasmids as above, except 2.5 μg of pDEST12.2-DDC was used and AR was excluded. Cells were then similarly treated with androgen and bicalutamide. Transfection efficiency was normalized with the *Renilla* luciferase pRL-TK vector for PC3 cells and for LNCaP cells RLU (relative light unit) values were obtained by normalizing to total protein concentration (RLU values are the means $\pm$ S.D. from triplicate determinations).

of interaction with the NTD (Figure 3). This indicates that the N-terminal of DDC (amino acids 1–333) may cause inhibition of binding to AR, especially with its N-terminus. Thus the presence of amino acids 1–333 could drastically lower the affinity of full-length DDC protein for the AR NTD bait in yeast, which may explain the absence of DDC clones with full-length cDNA insert in the screen. Also, the lack of an interaction between the DDC_{1-333} fragment and all AR domains suggests that the C-terminal region of DDC is solely responsible for interaction with both the NTD and LBD of AR. Although the DDC_{1-333} fragment contains an LXXLL motif (amino acids 153–157), which plays an essential role in co-activator interaction and co-activation function with steroid receptors, it does not appear to be involved in the DDC–AR interaction *in vitro*. This is not surprising since the LXXLL motif seems to be required for interaction with most steroid receptors except for AR [39], agreeing with the notion that AR–protein interactions are distinct than those of other steroid receptors.

The AR N-/C-terminal interaction is facilitated by several co-activators and is important for stabilization of bound ligand [3,40,41]. The ability of DDC to interact with both the N- and C-termini of AR suggests that it may play a role in modulating the folding of AR or facilitating the N-/C-terminal interaction. Examples of proteins that modulate AR activity in this manner include ARA70 [10], which stabilizes the ligand-bound receptor, and filamin, which facilitates the translocation of AR to the nucleus [42]. One component of the Hsp90 chaperone heterocomplex, BAG-1L (Bcl-2-associated athanogene-1 long isoform), also enhances ligand-dependent AR transactivation, probably through the appropriate folding of AR [43]. DDC, generally considered to be a cytosolic protein [44], probably increases AR transcriptional activity through one of these cytoplasmic processes. Also, since AR N-/C-terminal interactions may stabilize the AR dimer and promote its activity [39], the dual interaction of DDC with both these domains could play a role in AR dimerization. Through these events DDC may contribute to stabilize ligand binding or influence the subcellular distribution of AR, which would result in an overall increase in AR transcriptional activity. Moreover, the enhancement of AR transactivation seen with DDC co-transfection was significantly reduced when PC3 and LNCaP cells were treated with bicalutamide (Figure 7), suggesting that DDC exerts its effect on AR through a ligand-dependent pathway. Also, the increase in ligand-dependent AR activity seen with DDC co-transfection is not accompanied with an elevation in AR protein levels (Figure 5A). Further experimentation also showed that DDC co-expression does not alter AR protein levels at earlier time intervals (3, 6 and 12 h ligand treatment) in AR-transfected PC3 and non-transfected LNCaP cells (results not shown). Therefore, stabilization of AR protein can be ruled out as a possible mechanism for the observed increase in AR activity. The strong enhancement of transactivation for both AR and GR compared with the minimal increase in ER activity (Figures 5A and 6) may be due to the latter receptor's predominant nuclear localization even in the absence of its ligand [45]. Hence, any effect that DDC may have on steroid receptor protein in the cytoplasm would not alter ER activity profoundly.

DDC has been well characterized for its role as a PLP-requiring enzyme that catalyses the formation of DA from L-dopa and 5-hydroxytryptamine from L-5-hydroxytryptophan. Whether enzymic activity of DDC is involved in enhancing transcriptional activity of AR in PC3 and LNCaP cells has yet to be determined. *In vitro*, the PLP cofactor-binding site (amino acids 298–304) required for the enzymic activity of DDC is not necessary for interaction with AR, as the N-terminal DDC fragment (amino acids 1–333) does not interact with any AR domains (Figures 3B and 3C). However, *in vivo* the active enzyme, believed to consist of two $\approx$50-kDa homodimeric subunits (reviewed in [22]), may be important for indirectly regulating steroid receptor activity. One of the products of DDC, DA, has been shown to activate receptors such as ERα and the progesterone receptor in a ligand-independent manner in cultured cells. DA has no detectable affinity for intracellular steroid receptors. However, after DA binds to its membrane-bound receptor it may act by altering phosphorylation of either the steroid receptor itself or a specific transcription co-factor through a protein kinase A or other unknown pathway [46]. There is no information on the ability of DA to enhance AR transactivation or whether PC3 cells express the D1 and/or D5 DA receptors that are involved in stimulating steroid receptor transcriptional activity [46,47]. However, in HeLa cells (previously suggested to express functional D1 subtype DA receptors [28]) transfected with pAR6, DA treatment (0–100 μM) did not result in enhancement of AR transactivation (results not shown). Similarly, increasing concentrations of 5-hydroxytryptamine (0–100 nM) in pAR6-transfected PC3 cells, known to express 5-hydroxytryptamine

receptors [48], did not result in increased AR activity (results not shown). These observations suggest that the enhancement of AR transcriptional activity seen with high levels of DDC expression may not require synthesis of its neurotransmitter products.

It is becoming more apparent that a number of cancers are characterized by an extremely high level of DDC activity [49]. This enzyme has also been reported to be an excellent tumour marker for NE cell differentiation [50]. A recent report has shown that DDC expression and enzymic activity is markedly increased in prostates of NE-cell-transformed transgenic mice compared with normal prostates [23]. DDC expression was also detected in chromogranin A-positive human prostate adenocarcinoma cells, suggesting that it may be a biomarker of prostate cancer with NE differentiation. Increases in NE cell content occur during progression of prostate cancer and is often an indication of androgen independence or increased aggressiveness of the disease [51]. The rise in NE cells could result from the differentiation of transformed exocrine epithelial cells, which may still retain active AR and contain elevated levels of DDC. Increased DDC expression and subsequent enhancement of AR activity may cause these adenocarcinoma cells to have a lower androgen threshold for AR activation, leading to growth promoting effects under conditions where androgen levels are limited. This scenario seems to be supported by our observation that higher concentrations of bicalutamide were required to block androgen-stimulated AR transcriptional activity in PC3 and LNCaP cells transfected with DDC (Figure 7). Therefore, in hormone-refractory prostate cancer, increased expression of DDC could diminish the efficacy of anti-androgen treatment in blocking residual AR activity. Also, an alternative mechanism proposed to explain AR activity in androgen-independent prostate cancer is AR mutations that result in enhanced responses to other hormones. We found that the non-androgenic hormones E_2 and progesterone were able to weakly transactivate the mutant AR in LNCaP cells (using pARR3-tk-Luc reporter and hormone concentration of 10 nM), but transfection of DDC mildly enhanced AR activity only with the E_2 treatment (results not shown).

In conclusion, we have demonstrated that the RTA yeast two-hybrid system can be used to detect novel AR interacting proteins. Furthermore, it was shown that L-dopa decarboxylase can bind AR and enhance its transcriptional activity. DDC also enhanced GR transactivation, suggesting that it may play a role in regulating the activity of other steroid receptors. As there is evidence of DDC down-regulation by androgen [52] and its expression is elevated with NE differentiation, it may serve as an important regulator of AR transcriptional activity, during progression of prostate cancer to androgen independence. Further studies will reveal the mechanism of how DDC enhances AR activity and the role of this regulation in prostate cancer.

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REFERENCES

1 Green, S. and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. **4**, 309–314
2 Fang, Y., Fliss, A. E., Robins, D. M. and Caplan, A. J. (1996) Hsp90 regulates androgen receptor hormone binding affinity *in vivo*. J. Biol. Chem. **271**, 28697–28702
3 He, B., Kemppainen, J. A., Voegel, J. J., Gronemeyer, H. and Wilson, E. M. (1999) Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH_2-terminal domain. J. Biol. Chem. **274**, 37219–37225
4 Kuil, C. W., Berrevoets, C. A. and Mulder, E. (1995) Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. J. Biol. Chem. **270**, 27569–27576
5 Beato, M., Chavez, S. and Truss, M. (1996) Transcriptional regulation by steroid hormones. Steroids **61**, 240–251
6 Nelson, C. C., Hendy, S. C., Shukin, R. J., Cheng, H., Bruchovsky, N., Koop, B. F. and Rennie, P. S. (1999) Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. Mol. Endocrinol. **13**, 2090–2107
7 Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S. and Tung, L. (1996) Nuclear receptor coactivators and corepressors. Mol. Endocrinol. **10**, 1167–1177
8 Bevan, C. L., Hoare, S., Claessens, F., Heery, D. M. and Parker, M. G. (1999) The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol. Cell. Biol. **19**, 8383–8392
9 Fronsdal, K., Engedal, N., Slagsvold, T. and Saatcioglu, F. (1998) CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J. Biol. Chem. **273**, 31853–31859
10 Yeh, S. and Chang, C. (1996) Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc. Natl. Acad. Sci. U.S.A. **93**, 5517–5521
11 Petre, C. E., Wetherill, Y. B., Danielsen, M. and Knudsen, K. E. (2002) Cyclin D1: mechanism and consequence of androgen receptor co-repressor activity. J. Biol. Chem. **277**, 2207–2215
12 Lu, M. L., Schneider, M. C., Zheng, Y., Zhang, X. and Richie, J. P. (2001) Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J. Biol. Chem. **276**, 13442–13451
13 Truica, C. I., Byers, S. and Gelmann, E. P. (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res. **60**, 4709–4713
14 Hittelman, A. B., Burakov, D., Iniguez-Lluhi, J. A., Freedman, L. P. and Garabedian, M. J. (1999) Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. EMBO J. **18**, 5380–5388
15 Markus, S. M., Taneja, S. S., Logan, S. K., Li, W., Ha, S., Hittelman, A. B., Rogatsky, I. and Garabedian, M. J. (2002) Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus. Mol. Biol. Cell **13**, 670–682
16 Ueda, T., Bruchovsky, N. and Sadar, M. D. (2002) Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J. Biol. Chem. **277**, 7076–7085
17 McEwan, I. J. and Gustafsson, J. (1997) Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc. Natl. Acad. Sci. U.S.A. **94**, 8485–8490
18 Hirst, M., Ho, C., Sabourin, L., Rudnicki, M., Penn, L. and Sadowski, I. (2001) A two-hybrid system for transactivator bait proteins. Proc. Natl. Acad. Sci. U.S.A. **98**, 8726–8731
19 Ichinose, H., Kurosawa, Y., Titani, K., Fujita, K. and Nagatsu, T. (1989) Isolation and characterization of a cDNA clone encoding human aromatic L-amino acid decarboxylase. Biochem. Biophys. Res. Commun. **164**, 1024–1030
20 Ichinose, H., Sumi-Ichinose, C., Ohye, T., Hagino, Y., Fujita, K. and Nagatsu, T. (1992) Tissue-specific alternative splicing of the first exon generates two types of mRNAs in human aromatic L-amino acid decarboxylase. Biochemistry **31**, 11546–11550
21 Berry, M. D., Juorio, A. V., Li, X. M. and Boulton, A. A. (1996) Aromatic L-amino acid decarboxylase: a neglected and misunderstood enzyme. Neurochem. Res. **21**, 1075–1087
22 Zhu, M. Y. and Juorio, A. V. (1995) Aromatic L-amino acid decarboxylase: biological characterization and functional role. Gen. Pharmacol. **26**, 681–696
23 Hu, Y., Ippolito, J. E., Garabedian, E. M., Humphrey, P. A. and Gordon, J. I. (2002) Molecular characterization of a metastatic neuroendocrine cell cancer arising in the prostates of transgenic mice. J. Biol. Chem. **277**, 44462–44474
24 Rao, M. A., Cheng, H., Quayle, A. N., Nishitani, H., Nelson, C. C. and Rennie, P. S. (2002) RanBPM: a nuclear protein that interacts with and regulates transcriptional activity of androgen receptor and glucocorticoid receptor. J. Biol. Chem. **277**, 48020–48027
25 Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E. and Boeke, J. D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. Proc. Natl. Acad. Sci. U.S.A. **93**, 10315–10320
26 Rennie, P. S., Bruchovsky, N., Leco, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Snoek, R., Hamel, A., Bock, M. E., MacDonald, B. S. et al. (1993) Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. Mol. Endocrinol. **7**, 23–36
27 Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikstrom, A. C., Gustafsson, J. A. and Yamamoto, K. R. (1986) Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell **46**, 389–399

28 Smith, C. L., Conneely, O. M. and O'Malley, B. W. (1993) Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc. Natl. Acad. Sci. U.S.A. **90**, 6120–6124

29 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn, Cold Spring Harbor Press, Cold Spring Harbor, NY

30 Snoek, R., Rennie, P. S., Kasper, S., Matusik, R. J. and Bruchovsky, N. (1996) Induction of cell-free, *in vitro* transcription by recombinant androgen receptor peptides. J. Steroid Biochem. Mol. Biol. **59**, 243–250

31 Sato, N., Sadar, M. D., Bruchovsky, N., Saatcioglu, F., Rennie, P. S., Sato, S., Lange, P. H. and Gleave, M. E. (1997) Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. J. Biol. Chem. **272**, 17485–17494

32 Tzamarias, D. and Struhl, K. (1994) Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature (London) **369**, 758–761

33 Boeke, J. D., Trueheart, J., Natsoulis, G. and Fink, G. R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154**, 164–175

34 Jenster, G., van der Korput, H. A., Trapman, J. and Brinkmann, A. O. (1995) Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J. Biol. Chem. **270**, 7341–7346

35 Kanaoka, Y., Kimura, S. H., Okazaki, I., Ikeda, M. and Nojima, H. (1997) GAK: a cyclin G associated kinase contains a tensin/auxilin-like domain. FEBS Lett. **402**, 73–80

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

37 Veldscholte, J., Berrevoets, C. A., Brinkmann, A. O., Grootegoed, J. A. and Mulder, E. (1992) Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. Biochemistry **31**, 2393–2399

38 Zhao, Y., Goto, K., Saitoh, M., Yanase, T., Nomura, M., Okabe, T., Takayanagi, R. and Nawata, H. (2002) Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment. Identification of the p102 U5 small nuclear ribonucleoprotein particle-binding protein as a coactivator for the receptor. J. Biol. Chem. **277**, 30031–30039

39 Ting, H. J., Yeh, S., Nishimura, K. and Chang, C. (2002) Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc. Natl. Acad. Sci. U.S.A. **99**, 661–666

40 Moilanen, A. M., Karvonen, U., Poukka, H., Yan, W., Toppari, J., Janne, O. A. and Palvimo, J. J. (1999) A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins. J. Biol. Chem. **274**, 3700–3704

41 Ikonen, T., Palvimo, J. J. and Janne, O. A. (1997) Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J. Biol. Chem. **272**, 29821–29828

42 Ozanne, D. M., Brady, M. E., Cook, S., Gaughan, L., Neal, D. E. and Robson, C. N. (2000) Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol. Endocrinol. **14**, 1618–1626

43 Froesch, B. A., Takayama, S. and Reed, J. C. (1998) BAG-1L protein enhances androgen receptor function. J. Biol. Chem. **273**, 11660–11666

44 Poulikakos, P., Vassilacopoulou, D. and Fragoulis, E. G. (2001) L-DOPA decarboxylase association with membranes in mouse brain. Neurochem. Res. **26**, 479–485

45 Greene, G. L. and Press, M. F. (1986) Structure and dynamics of the estrogen receptor. J. Steroid Biochem. **24**, 1–7

46 Mani, S. K., Allen, J. M., Clark, J. H., Blaustein, J. D. and O'Malley, B. W. (1994) Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. Science **265**, 1246–1249

47 Apostolakis, E. M., Garai, J., Fox, C., Smith, C. L., Watson, S. J., Clark, J. H., O'Malley, B. W., Conneely, O. M., Jongsma, J., Oomen, M. H. et al. (1996) Dopaminergic regulation of progesterone receptors: brain D5 dopamine receptors mediate induction of lordosis by D1-like agonists in rats. J. Neurosci. **16**, 4823–4834

48 Jongsma, J., Oomen, M. H., Noordzij, M. A., Romijn, J. C., van Der Kwast, T. H., Schroder, F. H. and van Steenbrugge, G. J. (2000) Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines. Prostate **42**, 34–44

49 Watanabe, H., Imaizumi, M., Ojika, T., Abe, T., Hida, T. and Kato, K. (1994) Evaluation of biological characteristics of lung cancer by the human 28 kDa vitamin D-dependent calcium binding protein, calbindin-D28k. Jpn. J. Clin. Oncol. **24**, 121–127

50 Gazdar, A. F., Helman, L. J., Israel, M. A., Russell, E. K., Linnoila, R. I., Mulshine, J. L., Schuller, H. M. and Park, J. G. (1988) Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res. **48**, 4078–4082

51 Cohen, R. J., Glezerson, G. and Haffejee, Z. (1991) Neuro-endocrine cells – a new prognostic parameter in prostate cancer. Br. J. Urol. **68**, 258–262

52 Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L. and Lin, B. (2002) The program of androgen-responsive genes in neoplastic prostate epithelium. Proc. Natl. Acad. Sci. U.S.A. **99**, 11890–11895

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