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14. ABSTRACT

Too many prostrate-cancer treatments, especially those relying on the suppression of androgen, eventually fail to slow the advance of the disease. One explanation for this situation is the absence of any systematic knowledge on the role and function of the androgen receptor (AR) in the course of prostrate cancer development. Recent findings indicate that the AR is the key master regulator that determines disease progression to androgen independence, which ultimately contributes to death from prostate cancer. During the first year of this grant funding, we concentrated our efforts on the understanding of ligand-independent AR signaling in cells, which are resistant to ablation manipulations. Thus, we defined the role of protein kinase A pathway CREB as a ligand-independent mechanism in AR-mediated transcription at the prostate-specific antigen locus. We also found that certain coactivators mediate the interaction between the amino- and carboxyl-termini of the androgen receptor. Unexpectedly, however, in our latest work we demonstrated that although androgen ablation resistant cells are dependent on AR signaling for PSA expression, the AR was not recruited to the PSA enhancer or promoter sites where it normally mediates PSA expression. This result was published this month in Cancer Research. We are in the process of unraveling the mechanism of this interesting finding. Armed with a deeper knowledge of the hormonal and receptor requirements as well as mechanisms associated with prostrate cancer growth and expansion, we can develop therapies that prolong lives. Understanding the behavior of the androgen receptor is a first step in that quest.

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Introduction:

Treatment of advanced PCa often targets components of the AR signaling axis with small molecules that cause androgen ablation, thus inhibiting tumor growth. For patients who are either diagnosed with, or subsequently develop metastatic disease, the only targeted treatment option is androgen ablation [i.e. orchiectomy, treatment with LHRH agonists or antagonists and/or AR antagonists[1]]. Despite an initial positive response, androgen ablation is essentially palliative, and disease progression eventually ensues[2]. However, prostate cancer cells surviving after androgen ablation are in most cases sensitive to subsequent alternative hormone manipulations that depend on a functional AR. Resistance to androgen ablation is therefore not necessarily due to a loss of androgen sensitivity but probably develops as a consequence of a deregulated androgen signaling axis resulting from AR gene mutation or amplification, altered interactions of coregulatory molecules during transcription, or non-steroidal activation of the AR by growth factors and cytokines [reviewed in[3-6]]. In most cases such 'androgen-independent' signaling may simply reflect the ability of the AR to signal at ablation levels of androgen due to DHT hypersensitivity of the signaling mechanism. Altering AR/cofactor ratios, in turn, may bring this, about. Moreover, proof-of-principle that aberrant AR signaling may be sufficient to cause PCa was provided recently by the introduction of a mutant AR into transgenic mice. Mice carrying the mutant but not a wild-type AR transgene, subsequently developed PCa in 100% of cases[7]; the mutant AR had higher than normal transcriptional activity in the absence of ligand and had increased sensitivity to coregulators.

A note on terminology is appropriate. Traditionally, PCa that progresses after androgen ablation therapy (sometimes simply referred to as 'hormone treatment') has been termed 'androgen-independent', '-resistant' or '-refractile' and sometimes simply as 'hormone-independent', '-resistant' or '-refractile'. All these terms refer operationally to the recurrence of cancer after ablation therapy has failed, as measured by PSA rebound or clinical symptoms. Hormone ablation therapy may be either 'partial' (chemical or physical castration) or 'combined' (castration plus AR competitive inhibitor). However, in either case complete androgen-independence is probably never achieved in the tumors and AR signaling reappears after ablation failure. Consequently, such cancers are now being more correctly termed 'ablation-resistant' or 'castrate-resistant', which we believe less ambiguously reflects the situation in human and animal models.

Body:

In our original statement of work we had tentatively formulated a timeline of tasks. However, due to exciting novel experimental findings (see below), our priorities have changed somewhat. We have therefore followed 'our noses' into the mechanisms of the interesting findings that have resulted in a significant deviation from the originally defined order of tasks. The new findings, unpredictable 20 months ago when we prepared our DoD application, however still address our original aims and have led to a number of publications (see below and appendix).

In our original application we have formulated two specific aims:

Specific Aim #1: To identify non-steroidal pathways modulating AR activity.

In the present study, the effects of protein kinase A (PKA) signaling and its downstream factors on AR activity at the prostate specific antigen (PSA) gene were tested as a possible non-steroidal mechanism of AR-mediated transcription. Activation of PKA by forskolin resulted in enhanced androgen-induced expression of the PSA gene, an effect that was blocked by the AR antagonist, bicalutamide. Interestingly, when either p300 or CBP was overexpressed, PKA activation was sufficient to stimulate PSA promoter-driven transcription in the absence of androgen, which was not inhibited by bicalutamide. PKA activation did not significantly alter AR protein levels but significantly increased the phosphorylated form of its downstream effector, cAMP responsive element binding protein (CREB) in the presence of androgen. Furthermore, chromatin immunoprecipitation showed that the combination of androgen and forskolin increased phosphorylated CREB occupancy, which was accompanied by histone acetylation, at the putative cAMP responsive element located in the 5' upstream regulatory region of the PSA gene. Remarkably, mammalian-two-hybrid assay indicated that p300/CBP might bridge the interaction between AR and CREB, suggesting novel enhanceosomal cooperation. These results demonstrate an intriguing interplay between a signal transduction pathway, coactivator overexpression, and AR signaling as a possible combined mechanism of progression to androgen-independent prostate cancer. [8] [Appendix].

Specific Aim #2: To elucidate how non-steroidal signaling determines transcription complex compositions and histone code modifications in regulatory areas of AR target genes.

We have made substantial progress with this specific aim and have reported the following two studies (references at the end of each paragraph):

A. The androgen receptor (AR) mediates transactivation of target genes by acting as a dimer in which its amino-terminal domain (AR-NTD) interacts with its carboxyl-terminal, ligand-binding domain (AR-LBD) (N/C interaction). Here we assessed if and how AR N/C interaction relates to AR transactivation activity and how the p160 coactivator GRIP1 participates in both processes. The concentration of DHT needed for half-maximal N/C interaction was about 10-fold higher than for half-maximal transactivation, indicating a disparity between the two processes. Although a mutation of an LxxLL-like motif, ^{23}FQNLF^{27} to ^{23}FQNAA^{27}, in the AR-NTD abolished AR N/C interaction, it could be restored by the co-expression of the coactivator GRIP1. Co-expression of mutated forms of GRIP1, possessing alterations known to abolish either of two AR interaction domains, could not restore AR N/C interaction, suggesting that wild-type GRIP1 normally bridges the two AR domains. Although AR transactivation activity can proceed without AR N/C interaction, we propose that part of GRIP1 coactivation activity resides in its ability to bind both AR-NTD and -LBD, to stabilize the N/C complex and allow for secondary cofactors to be recruited more efficiently. Our

results also indicate that AR N/C interaction enhances but is not necessary for AR transactivation activity. [9] [Appendix].

B. Prostate specific antigen (PSA) expression is often used to measure AR activity in cells and prostate cancer progression in patients. In the present study we have compared AR activity using *PSA* and human male germ cell-associated kinase (*hMAK*), as read-outs in androgen-dependent LNCaP and androgen-independent C4-2B cells. As expected, very little *PSA* and *hMAK* expression were detected in LNCaP cells in the absence of androgens, while substantial expression of only *PSA* was observed in C4-2B cells under the same conditions. The addition of dihydrotestosterone (DHT) to the culture medium increased the expression of both genes in both cell types. Comprehensive chromatin immunoprecipitation (ChIP) analysis of the entire *PSA* locus and an androgen-response element (ARE) in *hMAK* unexpectedly revealed that AR was not occupying any site in the absence of DHT in either cell type. In line with the expression data, in the absence of DHT, histone acetylation and RNA polymerase II occupancy was substantial at the *PSA* locus in C4-2B but not in LNCaP cells. In the presence of DHT, AR was found to occupy mainly the enhancer region of *PSA* in both cell types, accompanied with increases in histone acetylation and RNA polymerase II occupancy. Although the AR was not directly involved in the androgen-independent expression of PSA in C4-2B cells, siRNA knockdown of AR significantly reduced PSA expression in both the presence and absence of DHT. In contrast, *hMAK* expression was decreased only in the presence of DHT after AR knockdown. We conclude that androgen-independent expression of PSA in C4-2B cells does not rely on the direct occupancy of the AR at the PSA locus, but is nevertheless affected indirectly via unknown AR-dependent mechanism(s) that influence the expression from some but not all AR target genes. [10] [Appendix].

Three review articles were written partially funded by the present grant (Prescott & Coetzee in press; Shen & Coetzee in press, Mulholland et al in press, all included in appendix).

Key Research Accomplishments:

1. AR signaling details were defined leading to an appreciation of the role of the AR in ablation resistant prostate cancer cells (three articles).
2. AR signaling was reviewed in three articles.

Reportable Outcomes:

As reported in six publications (see appendix).

Conclusions:

Overall final conclusions are pending.

Appendices:

Six articles referred to above are included.

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The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus

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Abstract

Androgen-independent prostate cancer is a lethal form of the disease that is marked by metastasis and rapid proliferation in its final stages. As no effective therapy for this aggressive tumor currently exists, it is imperative to elucidate and target the mechanisms involved in the progression to androgen independence. Accumulating evidence indicates that aberrant activation of androgen receptor (AR) via signal transduction pathways, AR gene mutation and/or amplification, and/or coregulator alterations may contribute to the progression of prostate cancer. In the present study, the effects of protein kinase A (PKA) signaling and its downstream factors on AR activity at the prostate-specific antigen (PSA) gene were tested. Activation of PKA by forskolin resulted in enhanced androgen-induced expression of the PSA gene, an effect that was blocked by the AR antagonist, bicalutamide. Interestingly, when either p300 or CBP was overexpressed, PKA activation was sufficient to stimulate PSA promoter-driven transcription in the absence of androgen, which was not inhibited by bicalutamide. PKA activation did not significantly alter AR protein levels but significantly increased the phosphorylated form of its downstream effector, cAMP responsive element-binding protein (CREB) in the presence of androgen. Furthermore, chromatin immunoprecipitation showed that the combination of androgen and forskolin increased phosphorylated CREB occupancy, which was accompanied by histone acetylation, at the putative cAMP responsive element located in the 5′ upstream regulatory region of the PSA gene. Remarkably, mammalian two-hybrid assay indicated that p300/CBP may bridge the interaction between AR and CREB, suggesting a novel enhanceosomal cooperation. These results demonstrate an intriguing interplay between a signal transduction pathway, coactivator overexpression and AR signaling as a possible combined mechanism of progression to androgen-independent prostate cancer.

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Introduction

Untreated prostate cancer progresses from androgen-dependent tumors that respond favorably to clinical therapies to recurrent, metastatic, androgen-independent tumors that are invariably fatal. Even at the latter, hormone-refractory stage, the tumor growth is dependent on a functional androgen receptor (AR) signaling pathway, as most androgen-independent prostate tumors continue to express AR and AR-regulated genes, such as prostate-specific antigen (PSA) (Buchanan *et al.* 2001). This evidence suggests that a critical process in tumor recurrence and progression may involve some compensatory regulation of AR activity in the low-androgen environment. Although the exact molecular basis for the progression to androgen-independent prostate cancer has yet to be defined, it is becoming increasingly clear that more than one specific mechanism may be involved. The proposed means of subverting growth control include AR gene mutation and amplification, coregulator alterations and androgen-independent activation of AR via various signal transduction pathways (Feldman & Feldman 2001). These mechanisms are not mutually exclusive but may in fact constitute compensatory measures that cooperate to establish androgen independence (Weber & Gioeli 2004).

Recent attention has focused on the hypothesis that the aberrant activation of AR through various signal transduction pathways plays an important role in tumor progression. Numerous studies indicate that AR signaling is affected by a diverse array of cytokines and growth factors, such as insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), interleukin 6 (IL-6) and forskolin, which act through a web of complex signal cascades, such as mitogen-activated protein kinase (MAPK), janus-activated kinase (JAK), signal transducers and activators of transcription (STAT), and protein kinase A (PKA) (Culig *et al.* 1994, Nazareth & Weigel 1996,

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Reinikainen *et al.* 1996, Sadar *et al.* 1999, Ueda *et al.* 2002, Jia *et al.* 2004). Of these pathways, PKA,, as activated by forskolin, is particularly interesting because of its possible ability to phosphorylate AR *in vivo* and stimulate the expression of the AR-regulated gene, PSA, in the absence of androgen (Sadar *et al.* 1999, Gioeli *et al.* 2002). Taken together, these observations suggest that PKA may modulate AR function, in part, by activating AR through phosphorylation in the absence of androgen. However, site-directed mutagenesis of AR phosphorylation sites has shown that the transcriptional activity of AR may not be affected by the post-translational modification (Gioeli *et al.* 2002). Thus, it is plausible that other mechanisms downstream of the PKA pathway are involved in the activation of AR-regulated genes.

One such mechanism that is downstream of the PKA pathway is the phosphorylation of cAMP responsive element-binding protein (CREB). CREB is a conceivable candidate for mediating PKA effects on AR-regulated transcription due to the presence of a putative cAMP responsive site (CRE) at the 5′ upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no reports have yet confirmed this phenomenon, it is a striking association in view of the PKA-induced transcription of PSA and the mechanism of CREB activity. PKA stimulation by cAMP, which is synthesized by forskolin-activated adenyl cyclase, leads to the phosphorylation of CREB at serine 133 (Ser 133), located within a transcriptionally critical region called kinase-inducible domain (KID) (Mayr & Montminy 2001). Phosphorylation of Ser 133 promotes binding of CREB to CRE and recruitment of the coactivator histone acetylase (HAT) paralogs CREB-binding protein (CBP) and p300 (Shaywitz & Greenberg 1999). The recruitment of CBP/p300 may specifically enhance the transcriptional potential of CREB by acetylating histones around the CRE and/or bridging the interaction between CREB and components of the basal transcription machinery (Nakajima *et al.* 1996, Korzus *et al.* 1998).

Interestingly, the physical bridging function of CBP/p300 and their HAT activities are both fundamentally important features that are also shared in the transcription activation modulated by nuclear hormone receptors, including the AR. Compelling studies suggest that, in the specific case of PSA gene transcription, CBP/p300 may not only bridge the interaction between AR and the basal transcription apparatus but also mediate the contacts between AR/coactivator complexes bound to the promoter and enhancer regions, thus forming 'enhanceosomes' (Shang *et al.* 2002, Louie *et al.* 2003). This theory of communication between 5′ regulatory elements is especially intriguing because of its implication that CBP/p300 could possibly mediate interactions between different transcription factors, such as AR and CREB, that share coactivators in common. Furthermore, another inference of the theory is that coactivators may play a crucial role in coordinating the increased transcriptional activity of AR in low-androgen environments by assembling stable, higher-order complexes. Indeed, transcriptional synergy among nuclear receptor coactivators via ternary complex formation has been documented (Lee *et al.* 2002). This also suggests that increased expression of coactivators may be an important mechanism that could compensate for the androgen-depleted state of advanced prostate cancer.

In the present study, the effects of PKA signaling and its mechanism of action on AR-regulated transcription at the PSA gene were investigated. The experiments confirmed that PSA gene expression was enhanced by PKA activation and demonstrated a novel mechanism of CREB and AR cooperation through CBP/p300. The evidence illustrated here presents a possibility of a unique cross-talk mechanism between AR and PKA signaling pathways.

Materials and methods

Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA, USA); it was used between passages 19 to 24. Cos 7 cells were also obtained from the ATCC and grown in OPTI-MEM (Invitrogen) supplemented with 5% (v/v) heat-inactivated FBS; they were used between passages 12 to 16. 5α-Dihyrotestosterone (DHT) was purchased from Sigma-Aldrich (St Louis, MO, USA). H-89 was purchased from Calbiochem (San Diego, CA, USA). Bicalutamide was obtained from Astra Zeneca. Forskolin was purchased from Sigma-Aldrich.

Transient transfection and luciferase detection

LNCaP cells (5×10^4 cells/well) in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS (Gemini) were plated in 96-well plates for 3 days. Cells were then transfected with reporter PSA-luc (100 ng/well; pGL3_PSA540), pCMV-300 or pCMV-CBP (50 ng/well; Dr T-P Yao, Duke University, Durham, NC, USA), and/or pCAT-basic (50 ng/well; Promega), a negative control to balance out the total amount of DNA, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. pGL3-PSA540-enhancer

(PSA-luc) is a mammalian expression vector that contains firefly luciferase linked to the androgen-responsive 548 bp PSA promoter region (−541 to +7) preceded by 1450 bp of the PSA enhancer region (−5322 to −3873) provided by Bristol-Myers Squibb (Princeton, NJ, USA). After transfection, cells were grown in phenol red-free RPMI 1640 containing 0·5% charcoal/dextran-stripped FBS with DHT, forskolin, bicalutamide and/or H-89 as indicated. After additional 30-h incubation, the cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity with the Promega kit, according to the manufacturer's protocol, and measured on a Dynex MLX Microtiter Plate Luminometer (Chantilly, VA, USA). Relative luciferase units (RLU) are shown as the means ± S.D. of quadruplicate wells. Total protein concentrations of the extracts were assayed with the Bio-Rad Protein Assay Kit (Hercules, CA, USA), according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices EMax Microplate Reader (Sunnyvale, CA, USA). No significant differences in total protein concentrations were observed among the different wells within a given experiment.

Chromatin immunoprecipitation (ChIP) assays

LNCaP cells (5×10^6 cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS (Gemini) for 3 days. Cells were treated with DHT and/or forskolin for 2 h, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The cells were washed twice with ice-cold PBS and harvested by scraping and centrifugation at 3000 ***g*** for 5 min. The cell pellets were resuspended in 0·5 ml lysis buffer (1% SDS, 10 nM EDTA, 50 nM Tris–HCl pH 8·0, with 1 × complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA)) and incubated for 20 min on ice. The cell lysates were sonicated at setting 4 on a Branson Sonifier Cell Disruptor 185 for 10 s (Fisher Scientific, Los Angeles, CA, USA). The sonication was repeated five times (with 1-min incubations on ice between sonications), and insoluble materials were removed by centrifugation at 15 500 ***g*** for 10 min. For each immunoprecipitation, 100 µl supernatant containing soluble chromatin was diluted 10-fold in dilution buffer (0·01% SDS, 1·1% Triton X-100, 1·2 mM EDTA, 16·7 mM Tris–HCl (pH 8·0), 167 mM NaCl and 1 × protease inhibitor cocktail). After preclearing with 75 µl of protein G–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4 °C for 1 h, the supernatant was immunoprecipitated by incubating at 4 °C overnight with 25 µl anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 10 µl anti-CREB, 10 µl anti-phospho CREB, 5 µl anti-dimethyl H3-K4 and 5 µl anti-AcH3 (Upstate Biotechnology, Lake Placid, NY, USA). Immune complexes were obtained by incubating with 50 µl protein G–Sepharose at 4 °C for 1 h. Immunoprecipitates were sequentially washed for 5 min each in low-salt wash buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8·0) and 150 mM NaCl), high-salt wash buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8·0) and 500 mM NaCl), LiCl wash buffer (0·25 M LiCl, 1% IGEPAL CA 630, 1% deoxycholate, 1 mM EDTA and 10 mM Tris–HCl (pH 8·0)) and TE buffer (twice). Washed beads were extracted with 250 µl elution buffer (1% SDS and 100 mM $NaHCO_3$) twice, the elution was combined, and the protein–DNA cross-linking was reversed by incubation at 65 °C overnight. Each sample was treated with 20 µg proteinase K (Gibco BRL, Grand Island, NY, USA) in proteinase K buffer (50 mM Tris–HCl (pH 6·5) and 10 mM EDTA) at 45 °C for 1 h. DNA was purified by phenol/chloroform extraction, precipitated by ethanol with glycogen, and resuspended in 100 µl H_2O. One percent of total soluble chromatin was processed in parallel without immunoprecipitation, and values obtained from this DNA were used as denominators to calculate immunoprecipitated DNA as percentage of input.

Real-time PCR of ChIP DNA

Immunoprecipitated DNA was analyzed by quantitative real-time PCR, as described previously (Jia *et al.* 2003). Briefly, triplicate PCRs for each sample were mixed with AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ, USA), forward and reverse primers and probe to be analyzed by Bio-Rad iCycler optical system. The primers and probes were as follows: enhancer forward, 5′-GCCTGGATCTGAGAGAGAT ATCATC-3′; reverse, 5′-ACACCTTTTTTTTTCTG GATTGTTG-3′; CRE forward, 5′-CTGGGCAAGC ACAATCTGA-3′; reverse, 5′-GTGTGATGCTGGAT GATGAGTG-3′; enhancer probe, 5′-6-FAM-TGC AAGGATGCCTGCTTTACAAACATCC-BHQ-1–3′; and CRE probe, 5′-6-FAM-CATGAAAGATGCCCC AGAGGCCTTG-BHQ-1–3′ (Biosearch Technologies, Novato, CA, USA). This assay provides a precise quantitation of target DNA and is based on the principle of fluorophore release from a self-quenching probe; the instrument measures the number of cycles (C_t) required for fluorescence to exceed a set threshold. A standard curve of known target DNA is constructed in parallel, from which the relative amount of target DNA in the sample is calculated. Values are presented as percentage input, which is analyzed at the same time. The precise quantitative nature of this analysis is superior to analyses commonly used by others that rely on semiquantitative end-point assessments of PCR bands on agarose gels.

Western blot analysis

LNCaP cells (6×10^5 cells/60 mm dish) were plated and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. After treatment with DHT, forskolin and/or H-89 as indicated, cells were harvested in 100 µl RIPA buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0·1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate and 0·2 mM Na_3VO_4 (pH 7·2)) that contained a cocktail of mammalian protease inhibitors. Total protein concentration of the protein extract was determined by the Bio-Rad Protein Assay Kit, according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices Emax Microplate Reader (Sunnyvale, CA, USA). Equal amounts of each protein extract were analyzed by SDS–PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with polyclonal anti-AR (AR-N20), anti-actin (Santa Cruz Biotechnology) and anti-CREB/anti-phospho-CREB (Upstate Biotechnology). HRP-conjugated anti-IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed by the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech), according to the manufacturer's protocol. Images are representative of three independent immunoblots and were analyzed by the Fluor-S Max MultiImager Quantification System (Bio-Rad).

Real-time RT-PCR of RNA

PSA mRNA was analyzed, as described previously, by quantitative RT–PCR. After treatment of the cells with DHT and/or forskolin, total cellular RNA was prepared and treated with RNase-free DNase I by the SV Total RNA Isolation System (Promega). A two-step RT–PCR method was employed, using the TaqMan Gold RT-PCR Kit (Applied Biosystems). In these analyses, the same probe and reverse primer located in exon 4 of the PSA gene were used for both the pre- and mature mRNA determinations (see below). The forward primer is located in intron 3 for pre-mRNA and in exon 3 for mature mRNA determinations. mRNA quantitation was performed by real-time RT-PCR. Thus, the primers and probes were as follows: PSA mRNA forward, 5′-GGCAGCATTGAACCAGAGGAG-3′; PSA reverse, 5′-GCATGAACTTGGTCACCTTCTG-3′; PSA probe, 5′-6-FAM-ATGACGTGTGTGCGCAAGTTC ACCC-BHQ-1–3′; GAPDH forward, 5′-GTC-ATGGG TGTGAACCATGAGA-3′; GAPDH reverse, 5′-GGT CATGAGTCCTTCCACGATAC-3′; and GAPDH probe, 5′-6-FAM-CAGCCTCAAGATCATCAGCAAT GCCTC-BHQ-1–3′ (Biosearch Technologies). Triplicate PCR reactions were conducted. GAPDH mRNA expression was analyzed in each sample in parallel wells. The results are represented as PSA/GAPDH mRNA ratios. Due to extensive DNase treatment of the RNA preparations (total RNA Isolation System; Promega), no significant genomic DNA contamination was apparent: 1. negative controls lacking reverse transcriptase were normally less than 1% of the experimental values (the value of each sample was adjusted by subtracting these negative values); 2. PCR analyses of promoter sequences gave negligible values.

Mammalian two-hybrid assay

We used reagents from the Clontech Mammalian Matchmaker Two-Hybrid Assay Kit to clone full-length CREB (pCF-CREB kindly provided by M R Montminy of the Salk Institute, La Jolla, CA, USA) into pM vector, and AR NTD and AR LBD into VP16 vector. Cos 7 cells (2×10^4 cells/well) in phenol red-free OPTI-MEM, containing 5% charcoal/dextran-stripped FBS (CSS), were plated in 96-well plates for 24 h. Cells were then transfected with reporter PGK1 (100 ng/well), pM CREB (20 ng/well), VP16 T Ag or VP16 AR NTD (20 ng/well), and GRIP1, p300 or CBP (50 ng/well), using Lipofect AMINE 2000 (Invitrogen), according to the manufacturer's protocol. After 4-h transfection, the lipofection mixture was removed and replaced with 5% CSS media with either ethanol (vehicle), DHT or forskolin as indicated. After 24-h incubation at 37 °C, the medium was removed, and cells were lysed with the passive lysis buffer (Promega). The extracts were assayed for luciferase activity as described above.

Results

Forskolin induces CBP/p300-mediated transcription of PSA-driven reporter

Previously, we reported that forskolin enhanced androgen-induced transcription of a PSA-driven reporter gene (Jia *et al.* 2003). In the present study, we sought to explore plausible mechanisms responsible for the observed effect, and considered downstream consequences of PKA activation. Our initial hypothesis was that the forskolin-activated PKA pathway may phosphorylate proteins involved in the AR signaling pathway, including AR and its coactivators. However, a recent study has demonstrated that the phosphorylation of AR does not affect its transcriptional activity (Gioeli *et al.* 2002). Thus, we examined whether coactivators that can be phosphorylated, such as CBP and p300 (Kitabayashi *et al.* 1995, Ait-Si-Ali *et al.* 1998), mediate the increased transcription through PKA signaling. We cotransfected LNCaP cells, which express a functional AR, with a luciferase reporter driven by PSA promoter and enhancer along with, either control, CBP or p300 plasmid (Fig. 1A). As previously described (Jia *et al.* 2003), transcription of

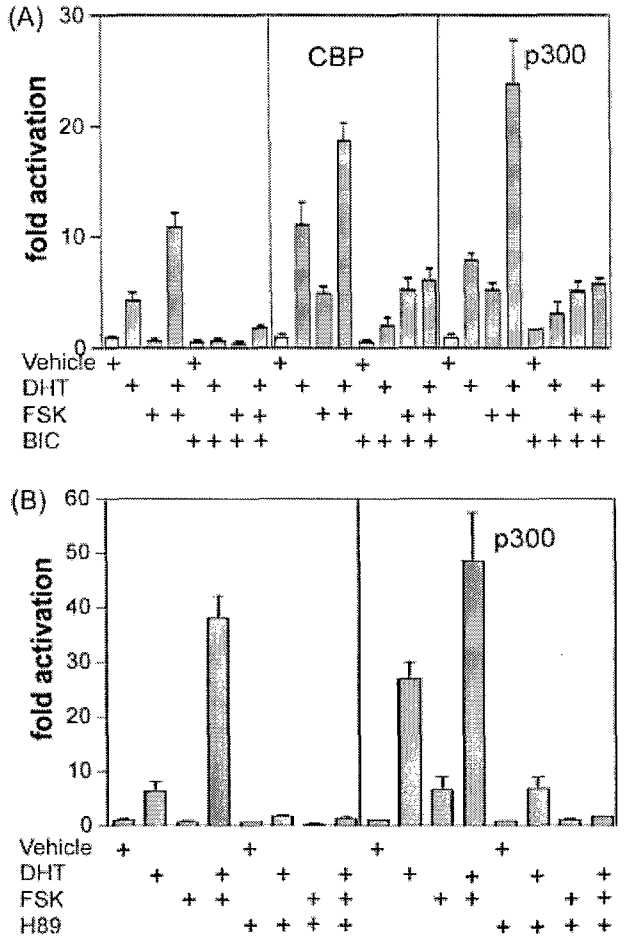

Figure 1 Forskolin enhances DHT-mediated and induces CBP/p300-mediated PSA transcription. (A) Transient transfection of LNCaP cells with AR-responsive reporter, PSA-luciferase, along with control (pCAT-basic), CBP or p300 expression plasmid. The transfected cells were treated with various combinations of ethanol (vehicle), 10 nM 5α-dihydrotestosterone (DHT), 50 μM forskolin (FSK) and/or 10 μM bicalutamide (BIC) as indicated. The values are represented as fold activation over the vehicle control (set as value, 1) in each transfection. (B) Transient transfection of LNCaP cells with PSA-luciferase with either pCAT-basic (as a control) or p300 expression plasmid. The transfected cells were treated with various combinations of vehicle, 10 nM DHT, 50 μM FSK and/or 10 μM H-89 (specific PKA inhibitor). The values are represented as fold activation over the vehicle control in each transfection. All values are means±S.E.M. of quadruplicate determinations, and the data are representative of three independent experiments.

PSA reporter induced by the AR ligand, DHT, was significantly enhanced by forskolin treatment. This effect was inhibited by the addition of the AR antagonist, bicalutamide, suggesting that AR signaling is necessary. Contrary to the earlier report by Sadar *et al.* (1999), we did not detect androgen-independent stimulation of PSA reporter transcription by forskolin treatment alone. Interestingly, however, when we overexpressed CBP or p300, we did observe forskolin-induced transactivation. Moreover, this induction was not inhibited by bicalutamide, indicating that AR does not mediate the androgen-independent stimulation. Moreover, when AR was blocked by bicalutamide, transcription stimulated by DHT and forskolin was reduced to the level of forskolin treatment alone. These results suggested that when CBP or p300 is overexpressed, a mechanism other than AR signaling is stimulated by the PKA pathway, and when combined with AR activity, it produces at least an additive, if not a synergistic, effect on PSA transcription. Next, we tested the effects of PKA pathway suppression with a specific inhibitor, H-89 (Fig. 1B). Surprisingly, when PKA signaling was blocked, both DHT- and DHT/forskolin-induced activities were abolished, indicating that the PKA pathway may be a crucial component of AR signaling in PSA gene transactivation. When p300 was overexpressed, forskolin stimulation was also inhibited by H-89, confirming that PKA signaling is responsible for the CBP/p300-mediated stimulation.

PKA activation enhances endogenous PSA gene expression and CREB phosphorylation

Due to the limitations of transient transfection assays, we were cautious in interpreting the transient transfection results; therefore, we sought to investigate the PKA effects *in vivo*. A quantitative analysis of PSA mRNA expression in LNCaP cells yielded similar results to the transient assays (Fig. 2A). Again, in contrast to the findings by Sadar (*et al.* 1999), we did not observe an androgen-independent production of PSA mRNA by forskolin treatment alone. PKA activation by forskolin, however, further stimulated DHT-induced PSA mRNA expression. This increased stimulation was significantly reduced but not completely abolished by bicalutamide (Fig. 2B), suggesting that inhibition of AR signaling does not eliminate the PKA component of the stimulation.

Next, we examined whether AR protein levels are affected by PKA activation (Fig. 3A). Addition of forskolin alone or in combination with DHT did not enhance AR expression. Taken together, these data strongly indicate that the enhanced expression of PSA by forskolin is not due to an increased expression or stabilization of AR by PKA signaling. Therefore, considering the results thus far and also the fact that CBP/p300 do not bind to DNA elements directly, we postulated that there may be another transcription factor that mediaties both androgen-dependent enhancement and androgen-independent induction of PSA transcription. An immediately apparent candidate was the CREB protein because of its ability to be activated by PKA signaling and its association with CBP/p300. We measured the endogenous expression of CREB in LNCaP cells and determined that the protein level did

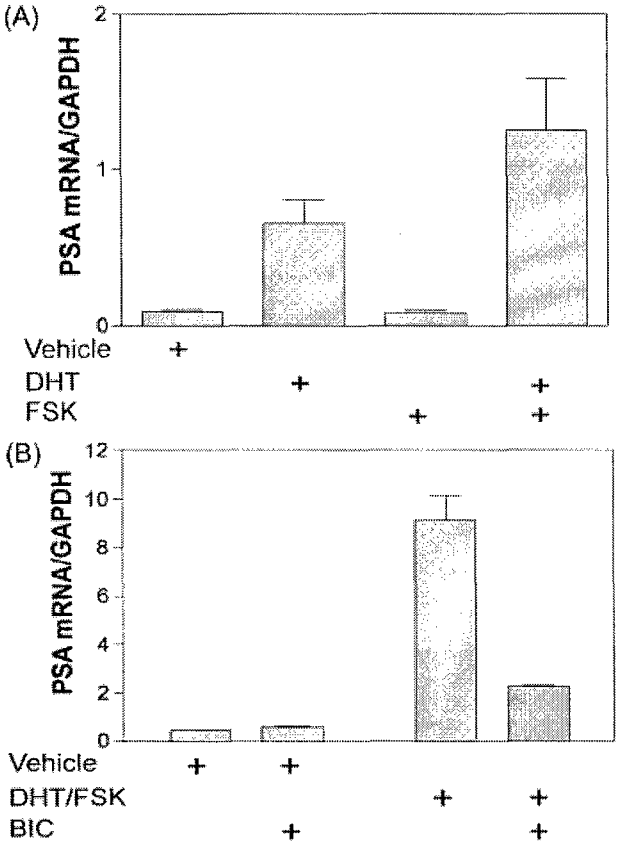

Figure 2 PKA activation enhances endogenous PSA gene expression. (A) Quantitative RT-PCR of PSA mRNA isolated from LNCaP cells after treatment of vehicle, DHT (ethanol), 10 nM DHT and/or 1 μM forskolin (FSK) for 16 h. The values are represented as a ratio of PSA mRNA to GAPDH. (B) PSA mRNA expression induced by DHT and FSK is inhibited by bicalutamide (BIC). LNCaP cells were treated with vehicle, DHT (ethanol), 10 nM DHT and 1 μM FSK, and/or 10 μM BIC for 16 h. The values are represented as means of ratios of PSA mRNA to GAPDH ±S.E.M. of triplicates, and are representative of five independent experiments.

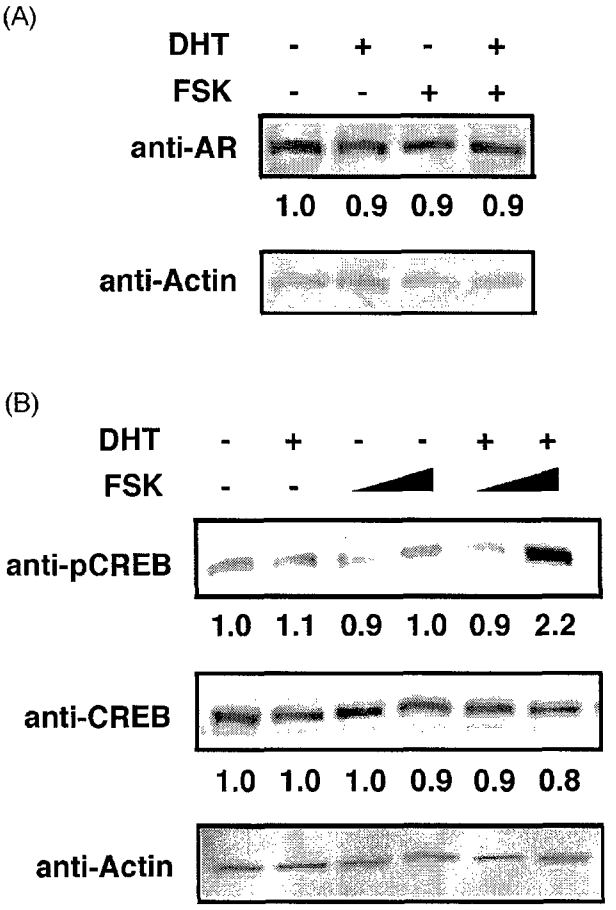

Figure 3 PKA activation does not affect AR protein expression but significantly increases CREB phosphorylation when combined with AR signaling. (A) LNCaP cells were treated with either 10 nM DHT or 1 μM forskolin (FSK), or both for 16 h and immunoblotted with anti-AR (N20) antibody. (B) For anti-CREB and antiphosphorylated CREB (pCREB) Western blot, LNCaP cells were treated with 10 nM DHT and/or 1 μM or 50 μM FSK for 10 min and immunoblotted with anti-CREB and anti-pCREB antibodies. Protein extracts in each case were loaded in equal amounts, as apparent by actin blotting. The number below each lane of protein expression indicates the relative intensity of each band normalized to corresponding actin expression level. The CREB and pCREB analyses are representative of four independent experiments.

not vary with DHT and/or forskolin exposure (Fig. 3B). However, the level of the phosphorylated form of CREB was significantly elevated by the DHT and forskolin combination. This evidence suggested the interesting possibility of a cross-talk between AR and PKA signaling that synergistically induces CREB activation, which in turn enhances androgen-dependent activation of PSA gene transcription.

CREB binds to CRE at the PSA 5′ regulatory region concomitant with histone acetylation

An additional rationale for CREB involvement in AR/PKA signaling at the PSA locus is provided by the fact that a putative CREB-binding site, CRE, is present at the 5′ upstream regulatory region of the PSA gene (Sadar *et al.* 1999). Although no characterization of this specific CRE exists in the literature, it is known to be a perfect 8 bp palindromic site (TGACGTCA) located at −3196/−3189 of the PSA gene (Fig. 4A). PSA CRE is apart from the AR binding sites located at the enhancer (−4148/−4134) and promoter (−170/−156). To investigate the role of CREB in AR-mediated transcription of the PSA gene, we chromatin immunoprecipitated (ChIP) for CREB binding and histone modifications at PSA CRE. Remarkably, quantitative analysis of CREB and phosphorylated CREB occupancy revealed that the

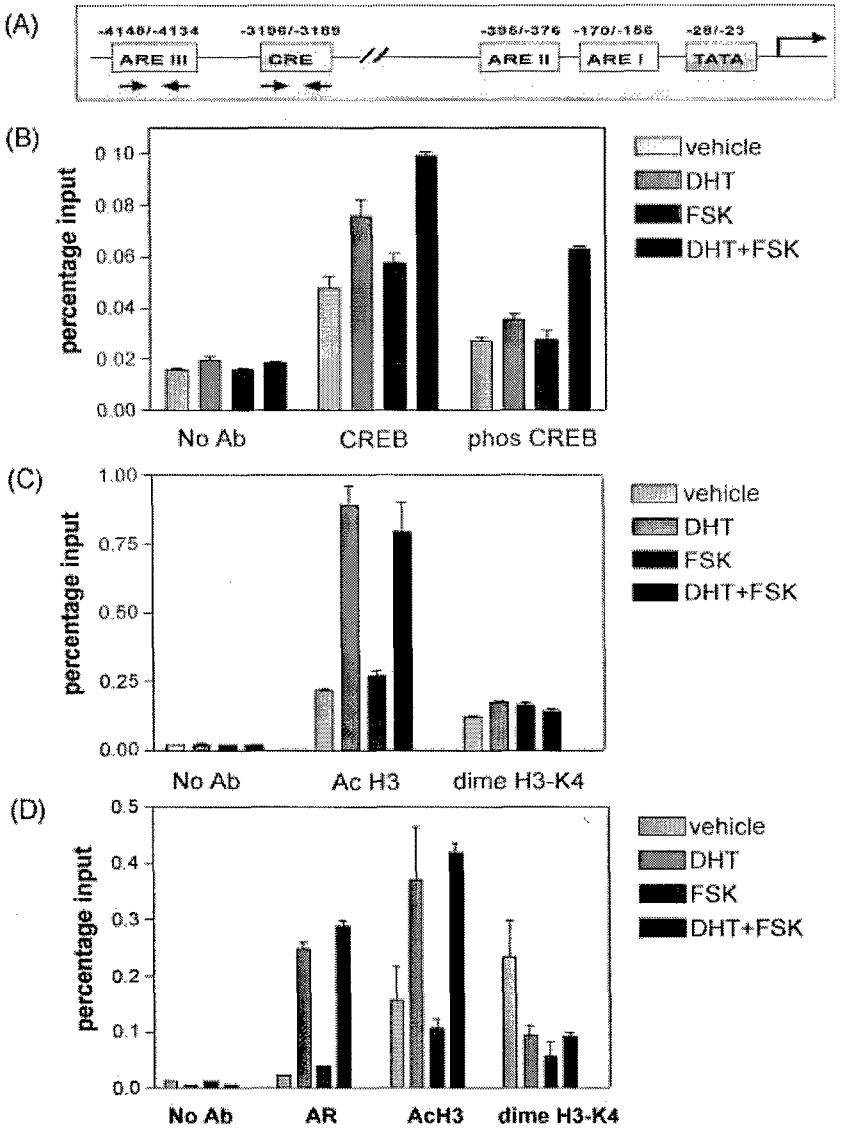

Figure 4 Phosphorylated CREB binds to CRE at the PSA 5′ regulatory region, which is concomitantly histone acetylated. (A) Real-time PCR primers and probes were designed around PSA enhancer ARE site (–4148/–4134) and CRE (–3196/–3189) for chromatin immunoprecipitation (ChIP) assay. Chromatin was sonicated to around a 500 bp fragment. (B) CREB and phosphorylated CREB occupancies at the PSA CRE site after treatment with vehicle, 10 nM DHT and/or 1 μM FSK. ChIP assay values are given as percentage of whole cell lysate (input). (C) Acetylation of histone H3 (Ac H3) and dimethylation of histone H3 at lysine 4 (dime H3-K4) at the CRE site measured by ChIP assay. The same conditions were used as above. (D) AR occupancy, acetylation of histone H3 and dimethylation of histone H3 at lysine 4 at the PSA enhancer ARE measured by ChIP assay. The same conditions were used as above. Values are means±S.E.M. of triplicate determinations and are representative of three independent experiments.

activated form of CREB was bound to the CRE only after DHT and forskolin exposure (Fig. 4B). Although the recruitment of activated CREB was low, the results were reproducible and in parallel with the low occupancy level of AR at the androgen response element (ARE) sites located in the promoter (Jia *et al.* 2003, Louie *et al.* 2003). We also examined acetylation of histone H3 (Fig. 4C) and H4 (data not shown) at the PSA CRE region and found that both were acetylated by DHT stimulation, suggesting that transcriptional potential is

increased at the DNA element. Although the combination of DHT and forskolin treatment also yielded histone acetylation, the absence of any significant change compared with the DHT treatment alone, taken together with the absence of acetylation with the forskolin exposure alone, may imply that acetylation of histones is a DHT-specific effect. Since we did not observe phosphorylated CREB occupancy with DHT treatment, these results raised the possibility that the immunoprecipitated CRE chromatin fragment contained the nearby ARE of the enhancer (~1 kb upstream of CRE) due to inefficient sonication. However, comparison of the dimethylation pattern of histone H3 at lysine 4 at the enhancer ARE (Fig. 4D) with that at the CRE (Fig. 4C) demonstrated that the two immunoprecipitated chromatin fragments were distinct. This is consistent with our previous finding that dimethylation pattern dynamically varies with transcription activation at the PSA AREs, but not at other regions (Kim *et al.* 2003). In addition, AR occupancy was not detected at the CRE, and CREB binding was not apparent at the enhancer ARE (data not shown), a finding which further supported the improbability of immunoprecipitating the same chromatin fragment. Therefore, it was apparent that an interesting cross-talk between AR and PKA signaling occurred at the CRE.

CBP/p300 mediates interaction between AR and CREB

Subsequently, we investigated whether a physical interaction between AR and CREB was responsible for the observed cross-talk. To test a possible AR and CREB interaction, we utilized the mammalian two-hybrid system in Cos 7 cells, which lack an endogenously expressed AR. While both AR and CREB can bind to CBP/p300 (Chrivia *et al.* 1993, Lundblad *et al.* 1995, Aarnisalo *et al.* 1998, Debes *et al.* 2002), no evidence of direct interaction between AR and CREB exists. Thus, we also examined the possibility that CBP/p300 physically bridges the interaction between AR and CREB. Full-length CREB was cloned into the GAL4 DNA-binding domain plasmid (pM-CREB), and N-terminal domain of AR was cloned into VP16 activation domain plasmid (VP16-AR NTD) (Fig. 5A). When CREB and a control vector (VP16 T Ag) were cotransfected in a mammalian two-hybrid assay (Fig. 5B), intrinsic activation by the forskolin treatment was observed, probably due to the transcriptional activation domain present in the CREB protein. As this activity does not reflect an interaction between CREB and the control protein, we determined this level to be the background, with which other interactions would be compared. Cotransfection of coactivators GRIP1 or p300 did not indicate bridging of interaction between CREB and the control protein. When CREB was cotransfected with AR NTD, no direct interaction was observed. Moreover, GRIP1 did not bridge the interaction between CREB and AR. However, cotransfection of p300 along with CREB and AR yielded a twofold increase in activity, suggesting that p300 can bridge the interaction between CREB and AR. This result is not due to p300's enhancing the transcriptional readout of GAL4 DNA-bound CREB, since p300 cotransfected with CREB and control protein did not produce a higher activity. Similar results were obtained when CBP was cotransfected (Fig. 5C), indicating that both CBP and p300 are able to bridge the interaction between CREB and AR. Notably, when CREB and ligand-binding domain of AR (AR-LBD) were coexpressed, CBP and p300 could not bridge the interaction (data not shown). This is consistent with the previously reported finding that CBP/p300 interacts with only the N-terminal domain of AR (Fronsdal *et al.* 1998). Therefore, the data presented suggest a possible indirect AR/CREB interaction mediated by CBP/p300.

Discussion

The AR plays a central role in the biology of prostate cancer, and androgen ablation therapy remains, after more than 50 years, the most effective treatment for advanced prostate cancer. However, prostate tumors treated with androgen ablation invariably progress to the therapy-resistant form that cannot be further treated with success. Although the tumors are androgen-independent at this final stage, a functional AR signaling pathway is maintained. Various molecular bases for the progression from androgen-dependent prostate cancer to the advanced disease have been proposed. Here, we illustrated a novel mechanism of cross-talk between AR and PKA signaling pathways, which cooperate to activate androgen-responsive gene transcription. Our data confirmed that PKA activation enhances androgen-dependent transcription from both transiently transfected and chromatin-integrated genes. A proposed consequence of PKA activation is thought to be the phosphorylation of AR, which in turn could render the protein more transcriptionally active. However, in addition to the conflicting result, indicating that PKA activation may actually dephosphorylate AR (Blok *et al.* 1998), a recent study showed that mutation of the phosphorylation sites does not have an effect on AR transcriptional activity (Gioeli *et al.* 2002). Moreover, the *in vivo* phosphorylation sites may regulate nuclear transport, and not transcription (Black *et al.* 2004). Our evidence suggests that PKA enhancement of androgen-mediated transcription is not due to increased AR expression or stabilization.

Contrary to the finding by Sadar *et al.* (1999), forskolin did not stimulate androgen-independent transcription in

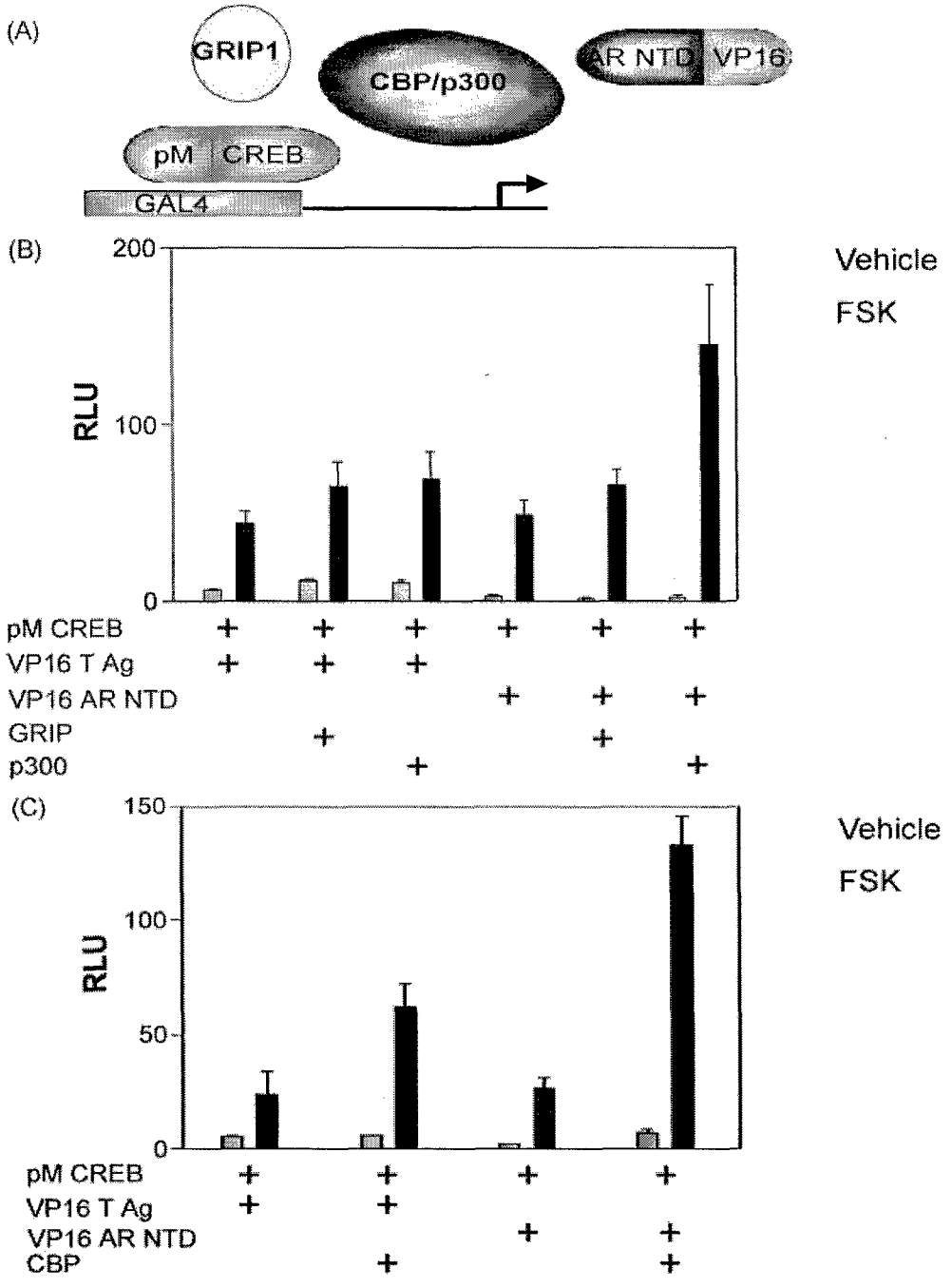

Figure 5 CBP/p300 mediates interaction between AR and CREB. (A) Mammalian two-hybrid assay scheme. Full-length CREB was inserted into GAL4 DNA binding pM plasmid, and N-terminal domain of AR (AR-NTD, aa 1–538) was cloned into VP16 activation domain plasmid. Luciferase reporter driven by GAL4 (PGK1), pM-CREB, VP16-AR NTD, GRIP1, CBP and/or p300 plasmids was cotransfected into Cos 7 cells lacking an endogenous AR. (B) p300, but not GRIP1, mediates the interaction between AR and CREB. Cos 7 cells were transfected with pM-CREB, VP16 T Ag (control) or VP16-AR NTD, GRIP1, and/or p300. Cos 7 cells were treated with vehicle or 10 μM forskolin (FSK). The values are represented as relative light units (RLU). (C) CBP mediates the interaction between AR and CREB. The same conditions as above were used in the experiment with CBP. Values are means±S.E.M. of quadruplicate determinations, and the data are representative of three experiments.

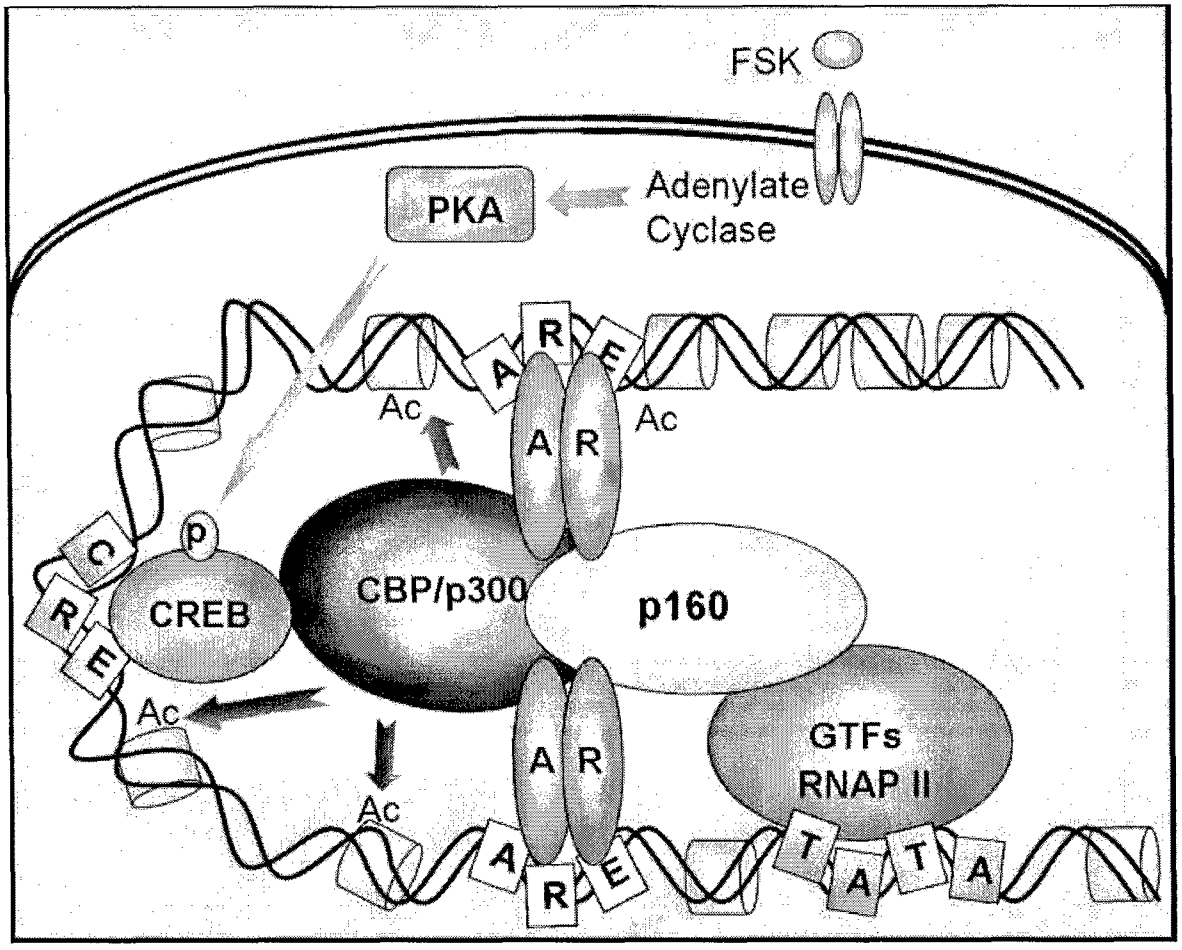

Figure 6 Proposed model of cross-talk between AR and PKA signaling pathway at the 5′ transcriptional control regions of the PSA gene. In this model based on the experiments illustrated in this study, PKA signaling phosphorylates and activates CREB, which binds to the PSA CRE site after DHT-induced AR signaling recruits CBP/p300, which mediates the interaction between AR and CREB. The recruitment of CBP/p300 acetylates the histones around the AREs and CRE. This higher-ordered enhanceosome assembly may stabilize the interaction with general transcription factors (GTFs) and RNA polymerase II (RNAP II), and thus increase the transcriptional potential at the PSA promoter.

our experiments. A possible explanation is that we tested early-passage (passages 19–24) LNCaP cells while the Sadar group used later-passage cells (passages 44–55). Taken together with the observation that CBP/p300 overexpression is sufficient to induce PKA-mediated transcription that is independent of DHT, our results suggest the interesting possibility that LNCaP cells may progressively express more CBP/p300 over time to compensate for the low-androgen environment. Activation of PKA signaling and increased expression of p300/CBP may be sufficient for progression of LNCaP cells toward androgen independence with respect to growth, gene expression and tumorigenicity. CBP and p300 are transcriptional coactivators that are fundamentally important in various signal-regulated transcription events. CBP was first identified as a protein that interacts with CREB, while p300 was isolated as a crucial cellular protein targeted by the adenovirus E1A oncoprotein (Whyte *et al.* 1989, Chrivia *et al.* 1993). Yet, CBP and p300 are highly related in structure and interact with a similar set of proteins, including p160 coactivators, general transcriptional apparatus, and nuclear hormone receptors (Giordano & Avantaggiati 1999). Since CBP/p300 does not bind to DNA elements directly, we examined whether another transcription factor was mediating the PKA effect. An attractive candidate was the CREB protein because of its association with CBP/p300 and its activation via the PKA pathway. Western blot data indicated that CREB phosphorylation is significantly increased by DHT and forskolin, corresponding to the observed enhancement of PSA transcription.

Moreover, ChIP assays demonstrated a novel occupancy of CREB at the PSA CRE site, which was

accompanied by histone acetylation. Interestingly, only the combination of DHT and forskolin treatment recruited activated CREB to CRE, further supporting a cross-talk between AR and PKA signaling. This phenomenon may reflect the significantly increased level of phosphorylated CREB induced by DHT and forskolin treatment of LNCaP cells, as evidenced by the Western blot data. Although the K_d for CREB binding to palindromic CREs is low (1 nM) compared with the nuclear concentration of the protein (100 nM), the CRE is generally less active when moved further upstream of the promoter (Shaywitz & Greenberg 1999). Thus, the increased availability of phosphorylated CREB may be a crucial event mediated by AR and PKA cross-talk. In addition, the mammalian two-hybrid data that suggest CBP/p300 is able to mediate the interaction between AR and CREB, taken together with our ChIP results, may indicate that AR and CREB form an enhanceosome at the 5′ regulatory regions of the PSA gene (Fig. 6). In this model, stimulation of androgen and the PKA pathway activates both AR and CREB, which recruit CBP/p300, forming a more stable tertiary interaction in an enhanceosome. As the specific recruitment of a complex with HAT activity to a promoter may play a critical role in overcoming the repressive effects of chromatin structure on transcription (Struhl *et al.* 1998), a higher-ordered assembly that recruits histone acetylation to the regulatory elements could increase the transcription potential. CREB may also function cooperatively with CBP/p300 to regulate AR activity, in part by enhancing the recruitment of TAF_{II} 130 to the promoter (Nakajima *et al.* 1997, Felinski & Quinn 1999).

As androgen and the AR play important roles in prostate cancer progression, understanding the factors involved in the regulation of androgen/AR action may provide molecular targets for prostate cancer treatment. Aberrant activation of the AR through the PKA signaling pathway may be responsible for the progression of prostate tumors to the rapidly proliferating, androgen-independent state. Further investigation of the PKA signaling effect on AR activity may yield valuable information potentially leading to viable therapies in the future.

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Short Communication

GRIP1 mediates the interaction between the amino- and carboxyl-termini of the androgen receptor

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Abstract

The androgen receptor (AR) mediates transactivation of target genes by acting as a dimer in which its amino-terminal domain (AR-NTD) interacts with its carboxyl-terminal, ligand-binding domain (AR-LBD) (N/C interaction). Here we assessed if and how AR N/C interaction relates to AR transactivation activity and how the p160 coactivator GRIP1 participates in both processes. The concentration of dihydrotestosterone needed for half-maximal N/C interaction was approximately 10-fold higher than for half-maximal transactivation, indicating a disparity between the two processes. Although a mutation of an LXXLL-like motif, ^{23}FQNLF$^{27} \rightarrow {}^{23}$FQNAA^{27}, in the AR-NTD abolished AR N/C interaction, it could be restored by the co-expression of the coactivator GRIP1. Co-expression of mutated forms of GRIP1, possessing alterations known to abolish either of the two AR interaction domains, could not restore AR N/C interaction, suggesting that wild-type GRIP1 normally bridges the two AR domains. Although AR transactivation activity can proceed without AR N/C interaction, we propose that part of the GRIP1 coactivation activity resides in its ability to bind both AR-NTD and -LBD, to stabilize the N/C complex and allow for secondary cofactors to be recruited more efficiently. Our results also indicate that AR N/C interaction enhances but is not necessary for AR transactivation activity.

Keywords: androgen receptor; coactivator; dimerization; domain interactions; transactivation; transcription.

The androgen receptor (AR) has three well-defined modular protein domains: an amino-terminal domain (NTD) involved principally in transcriptional activation, a DNA-binding domain (DBD) that is required for interaction with specific gene sequences, and a carboxyl-terminal ligand-binding domain (LBD) that mediates the specific interaction between the AR and its cognate ligands (Gelmann, 2002). Following ligand binding, the LBD undergoes an ordered structural rearrangement that results in the formation of a highly conserved protein-protein interaction surface known as activation function 2 (AF2). Early work indicated that the NTD interacts with the LBD in what has been referred to as AR N/C interaction, and that this is facilitated by coactivators (Ikonen et al., 1997). More recently, it was shown that AF2 of most nuclear receptors is occupied by LXXLL-like motifs [known as nuclear receptor (NR) boxes, where L is leucine and X is any amino acid] of coregulatory proteins, of which the p160 family is the most widely characterized (Ma et al., 1999). In contrast, AF2 of the AR is predominantly occupied by an LXXLL-like peptide, ^{23}FQNLF^{27} of the AR-NTD (He and Wilson, 2002). This interaction is thought to mediate AR homo-dimerization and to be important for AR function *in vivo*. Of essential importance are the following points: (i) AR-AF2 interacts specifically and with greater avidity with this single ^{23}FQNLF^{27} of the AR-NTD than with the NR boxes of p160 coactivators; and (ii) AR N/C interaction inhibits p160 coactivator recruitment to the AR-AF2 in the LBD (He et al., 2001).

We have previously shown through glutathione S-transferase (GST) pull-down assays that a p160 coactivator of AR, GRIP1, interacts with the AR-AF2 region via its LXXLL boxes situated approximately in the middle between the GRIP1 N- and C-termini (Ma et al., 1999), as well as with a C-terminal portion of the AR-NTD via the GRIP1 C-terminal domain (Irvine et al., 2000). This previously published evidence of physical protein/protein interaction between GRIP1 and the separate AR NTD and LBD domains has led us to hypothesize that GRIP1 may bridge AR N/C domains, thereby facilitating or stabilizing AR inter-domain communication, and to propose that the primary consequence of this facilitated bridging is increased AR transactivation activity.

In the present study we assessed whether AR N/C interaction and transactivation of target genes are mechanistically linked and how p160 coactivators participate in the two processes. Both processes are dependent on androgen (dihydrotestosterone, DHT), but to different extents (Figure 1). Concentrations of DHT necessary to yield half-maximal activity were approximately 1 and 10 nM DHT for transactivation activity and N/C interaction, respectively, indicating a disparity between the two processes. For example, at 1 nM DHT, transactivation activity proceeded at approximately half-maximal rates, while N/C interaction was barely detectable (Figure 1),

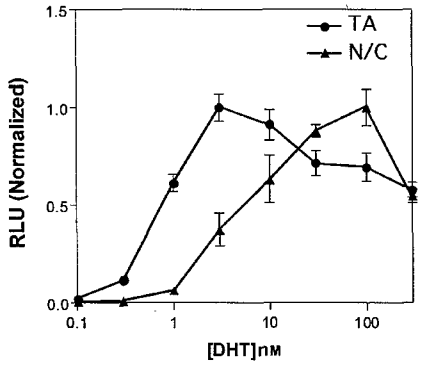

Figure 1 DHT dose-response curves of AR transactivation and N/C interaction.
COS7 cells (1×10^4 cells/3-mm well) were transfected with DNA-expressing AR constructs and luciferase reporters. Transfected cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS in the absence or presence of varying concentrations of DHT (0.1, 0.3, 1, 3, 10, 30, 100, and 300 nM). The data represent normalized RLU values, with the maximal activity of each curve set at 1.0. Results of the transactivation experiment represent the mean (±SEM) of 10 individually transfected wells, whereas the results of the N/C interaction experiment represent the mean (±SEM) of 5 individually transfected wells.
Methods: Transactivation of the full-length wild-type AR (pcDNA AR WT) activity was assayed using the AR responsive probasin-luc reporter. Full-length AR consisting of amino acids 1–919 was cloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) as previously published (Irvine et al., 2000). Probasin-luc (ARR3-tk-luc/tk81-PB3) is a mammalian expression vector that contains firefly luciferase linked to three copies of the androgen-responsive minimal rat probasin promoter (-244 to -96) ligated in tandem to the thymidine kinase (tk) enhancer element (Snoek et al., 1998; Kasper et al., 1999) provided by Dr. R.J. Matusik (The Vanderbilt Prostate Cancer Center, Nashville, TN, USA). N/C interaction of the wild-type AR NTD 1-538 VP16-AD fusion vector (VP16 AR-NTD WT) with the AR LBD 644-919 GAL4-DBD fusion vector (GAL4 AR-LBD) was assayed by measuring GAL4-responsive luciferase reporter (pGK1) as relative light units (RLU), using the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech, Palo Alto, CA, USA). COS7 cells (ATCC, Manassas, VA, USA) were maintained in Dubelco's modified Eagle medium (DMEM) hi-glucose media (USC/Norris Cancer Center Microchemical Core facility) that was supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) and used between passages 7 and 12. Cells were plated in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h in DMEM. Subsequent transient transfection of plasmids was conducted using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's suggested protocol. After transfection, cells were grown in Opti-MEM I reduced serum medium (Invitrogen) in the absence or presence of DHT (Sigma Chemical Co, St. Louis, MO, USA) for 24 h in the case of the mammalian two-hybrid assay, or 36 h in the case of the full-length AR transactivation assay. After incubation cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA) and extracts assayed for luciferase activity using the Promega Luciferase Assay System measured on a MLX Microtiter Plate Luminometer (Dynex, Chantilly, VA, USA).

providing evidence that the former can proceed without the latter. One caveat when comparing the data from the two assay systems is that the two-hybrid assay design used to measure N/C interaction may exclude effects of full-length receptor dimerization on DHT binding stability. Thus, while the data reported here represent the isolation of the N/C interaction event from the overall AR transactivation, more insight into this comparison will come with a better understanding of the role of N/C interaction in AR activation. The data are independently supported by the findings of the Brinkmann and Trapman group (Doesburg et al., 1997) who reached the same conclusions regarding a 10-fold disparity in DHT response of AR N/C interaction versus transactivation.

To understand better the mechanism(s) of AR N/C interaction, the role played by coactivators, and how different molecular interactions participate in AR transactivation activity, we next investigated interdomain interactions of both wild-type AR and a mutant AR that contains alterations known to affect the N/C interaction. When both wild-type AR-NTD and AR-LBD domains were co-expressed in cells in a mammalian two-hybrid assay, treatment with DHT resulted in strong interaction not explicable by activity of either fusion protein expressed with irrelevant partners (Figure 2A). This interaction was enhanced nearly 10-fold when wild-type GRIP1 was co-expressed, suggesting that GRIP1 may act to stabilize the AR-NTD and -LBD interaction, possibly via physically bridging the two AR domains. Recruitment of GRIP1 to GAL4 AR-LBD in the absence of an interacting AR-NTD resulted in near-negligible activity (Figure 2A). When AR-NTD containing the alteration ^{23}FQNLF^{27} → ^{23}FQNAA^{27} (VP16 AR-NTD FQNAA) was co-expressed with wild-type AR-LBD, treatment with DHT alone resulted in an N/C interaction that was also only marginally above the no-DHT background level, suggesting that the FQNLF motif is a key interaction surface for DHT-driven AR N/C interaction. However, DHT-dependent N/C interaction involving the ^{23}FQNAA^{27} mutant was dramatically restored when wild-type GRIP1 was co-expressed in the assay, suggesting that the enhancing effect of GRIP1 on N/C interaction could still operate in the presence of the altered AR FQNLF motif. The restored N/C interaction by GRIP1 cannot be attributed to artifacts of intrinsic GRIP1 coactivation activity, as GRIP1 recruitment to GAL4 AR-LBD alone did not result in appreciable activity. Therefore, the recovery of ^{23}FQNAA^{27} N/C interaction by GRIP1 is evidence that enhancement of the AR N/C interaction by GRIP1 is likely due to binding surface(s) in the AR-NTD other than the FQNLF motif.

To explore further the nature of the GRIP1 enhancement of AR N/C interaction, we reasoned that GRIP1 mutants lacking the ability to bind either the AR-NTD or AR-LBD would fail to properly function as a bridge between the domains, and would thus fail to enhance N/C interaction beyond that induced by DHT. We have previously published evidence demonstrating the physical interaction between two independent subdomains of GRIP1 and AR-LBD and AR-NTD through the use of mammalian two-hybrid assays, GST pull-down assays and mutational analyses (Ma et al., 1999; Irvine et al., 2000). To address the implications of these findings in the present experiments, we utilized two GRIP1 mutant constructs, each with impaired binding to one of the AR subdomains. The first contains a truncation that eliminates the C-terminal 341 amino acids [GRIP1 (1-1121)], thus losing the NID_{AF1} region previously shown to be nec-

essary for GRIP1/AR-NTD binding (Ma et al., 1999). The second has LXXLL→LXXAA alterations in NR boxes 2 and 3 (GRIP1 NR*), thereby eliminating the ability of GRIP1 to bind to AF2 in the AR-LBD (Ma et al., 1999). When the two mutant GRIP1 constructs were introduced individually into cells along with co-expressed wild-type AR-NTD and AR-LBD fragments, DHT-dependent N/C interaction was unaffected by the GRIP1 (1-1121) mutant but enhanced by the GRIP1 (NR*) mutant. The latter observation indicates that even in the absence of GRIP1/AR-LBD binding, the recruitment of GRIP1 to the transcription complex still occurs through GRIP1 associations with the AR-NTD in the context of DHT-induced AR N/C interaction. Furthermore, Western immunoblot analyses showed a small but consistent increase in the levels of AR-NTD fusion proteins in the presence of GRIP1 and GRIP1 mutants, suggesting stabilization of AR fragments by the p160 coactivator. Therefore, it is

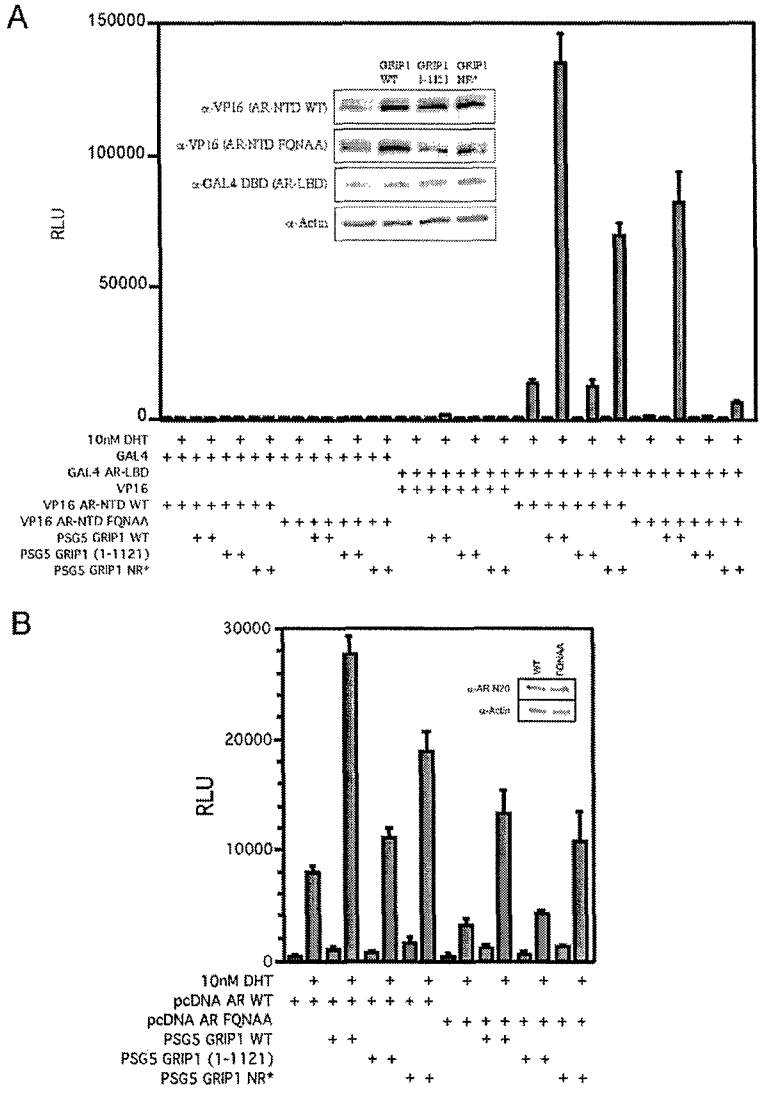

Figure 2 Effect of ^{23}FQNAA^{27} mutation on AR N/C interaction and full-length AR transactivation.
COS7 cells were transfected with DNA expressing AR constructs in the absence or presence of co-expressed wild-type GRIP1 (PSG5 GRIP1 WT) or mutant GRIP1 [PSG5 GRIP1 (1-1121) or GRIP1 NR*]. Transfected cells were incubated in the absence or presence of 10 nM DHT. Luciferase reporter activity is given in relative light units (RLU). Data shown are representative of three individual experiments. (A) Cells were transfected with DNA expressing fusion plasmids of VP16-AD with either wild-type AR NTD 1-538 (VP16 AR-NTD WT) or AR NTD 1-538 containing ^{23}FQNAA^{27} (VP16 AR-NTD FQNAA), along with GAL4-DBD fused with AR LBD 644-919 (GAL4 AR-LBD) in the absence or presence of wild-type or mutant GRIP1. N/C interaction was assayed using a transfected GAL4-responsive luciferase reporter (pGK1). Inset: Western blot data indicate the relative levels of the AR NTD and LBD fusion proteins in the presence of DHT and the GRIP1 constructs using antibodies directed against VP16-AD and GAL4-DBD epitopes. (B) Cells were transfected with DNA-expressing full-length constructs of wild-type AR (pcDNA AR WT) or AR containing ^{23}FQNAA^{27} (pcDNA AR FQNAA) in the absence or presence of wild-type or mutant GRIP1. AR transactivation was assayed using a transfected PSA promoter-driven luciferase reporter (PSA-luc). Values are mean±SEM of quadruplicate determinations and the data are representative of three independent experiments. Inset: Western blot data indicate the relative levels of the AR constructs in the presence of DHT (in the absence of transfected coactivators) using antibodies directed against an AR-NTD epitope.

plausible that the increased AR N/C interaction activity caused by GRIP1 in the mammalian two-hybrid assay involving wild-type AR fragments results from three mechanisms: (i) increased N/C interaction through GRIP1 bridging; (ii) increased recruitment of GRIP1 to the transcription complex; and (iii) stabilization of the AR-NTD fusion proteins by GRIP1.

Within the same assay conditions, results involving the AR-NTD FQNAA mutant were dramatically different (Figure 2A). While wild-type GRIP1 rescued the inhibited AR N/C interaction of the AR-NTD FQNAA mutant as previously noted, the two GRIP1 mutants were largely unable to do so, indicating that both AR-NTD and -LBD interacting domains of GRIP1 are necessary to achieve the putative bridging activity. Once again, the small apparent induction of AR N/C interaction observed in response to the co-expression of GRIP1 NR* mutant is likely due to the activity of GRIP1 NR* recruited to the small amount of AR-NTD engaged in the marginal N/C interaction involving the AR-NTD FQNAA mutant. It is clear that substantially lower AR N/C interaction activity occurred between AR-NTD FQNAA and AR-LBD in the presence of either of the GRIP1 mutants compared to the activity of wild-type AR-NTD.

We have schematically depicted the different molecular interactions involved in the AR N/C mammalian two-hybrid assay (Figure 3). Figure 3A–D illustrates the interactions involving wild-type AR-NTD, whereas Figure 3E–H, the interactions involving the AR-NTD ^{23}FQNAA^{27} mutant. We propose that wild-type GRIP1 stabilizes wild-type AR N/C interactions, thereby mediating coactivation (Figure 3B). However, mutant GRIP1 (1-1121) is unable to do so (Figure 3C), indicating that the LXXLL boxes of GRIP1 are unable to compete effectively against ^{23}FQNLF^{27} of the AR-NTD for AR-LBD binding. On the other hand, mutant GRIP1 (GRIP1 NR*) that is able to bind to the AR-NTD, but not to the AR-LBD, can presumably still mediate coactivation activity in the assay, albeit not to the level of wild-type GRIP1 (Figure 3D). The AR-NTD ^{23}FQNAA^{27} mutant (which is unable to mediate AR N/C interaction; see Figure 3E) can be bridged to AR-LBD by wild-type GRIP1, yielding high activity (Figure 3F), but not to the same extent with GRIP1 (1-1121) (Figure 3G), or GRIP1 NR* (Figure 3H), again probably due to the GRIP1 mutants lacking either one of their AR-interacting surfaces. Our results therefore indicate that AR N/C interaction can occur directly via the involvement of AR-NTD ^{23}FQNLF^{27}or indirectly via GRIP1 bridging.

We next examined the effects of GRIP1 and its mutants on wild-type and ^{23}FQNAA^{27} mutant AR transactivation activity. Co-expressed wild-type GRIP1 resulted in an approximately three-fold increase in DHT-dependent receptor activity (Figure 2B). When either of the two mutant GRIP1 constructs was co-expressed in place of wild-type GRIP1, the receptor transactivation activity was still enhanced over that of DHT stimulation alone, albeit modestly compared to wild-type GRIP1. The situation with AR FQNAA mutant was very similar and, importantly, substantial transactivation activity was observed for this AR mutant, even in the presence of mutant GRIP1 molecules. Most notably, these same GRIP1 mutants were unable to rescue N/C interaction activity between AR-NTD FQNAA and AR-LBD, as previously discussed (Figure 2A). Whereas coactivation of the FQNAA mutant AR by wild-type p160 coactivators was previously reported by the Wilson group (He et al., 2002), the present work demonstrates that this coactivator response may occur mechanistically through the restoration of impaired N/C interaction by coactivator molecules bridging AR domains. However, given that the AR FQNAA mutant maintains substantial transactivational competence, the rescue of its nearly completely impaired N/C interaction should be regarded as a mechanism that further contributes to, but is not critical for, transactivation. Thus, the disparity between levels of AR N/C interaction and AR transactivation activity reported here indicates that AR N/C interaction is not necessary for AR transactivation activity.

Figure 2 Methods: Mutations in the AR were constructed using the protocol published in the Stratagene Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). pcDNA AR FQNAA codes for full-length AR with the ^{23}FQNAA^{27} mutation. AR-NTD FQNAA codes for AR transactivation domain amino acids 1–538 containing the ^{23}FQNAA^{27} mutation. PSA-luc consists of firefly luciferase driven by the androgen-responsive 548-bp PSA promoter region (-541 to +7) preceded by 1450 bp of the PSA enhancer region (-5322 to -3873) (Bristol-Myers Squibb, Princeton, NJ, USA). Three GRIP1 expression constructs (a gift from the laboratory of Dr. Michael Stallcup) consisted of PSG5 GRIP1 coding for full-length GRIP1 amino acids 1–1462, PSG5 GRIP1 (1-1121) coding for a truncated form of the protein lacking activation domain 2, and PSG5 GRIP1 NR* coding for the full-length protein having the second and third LXXLL motifs in the NR-box domains mutated to LXXAA. In all experiments we balanced molar amounts of expression vectors with their empty counterparts as controls and balanced the nanogram amounts of total DNA with pCAT-basic, a eukaryotic promoter-less vector. Under these conditions, experimental and control situations contained identical concentrations of total DNA per transfection (i.e., ng/transfection) and an identical copy number of promoter elements (i.e., molar amounts). For Western immunoblot analyses COS7 cells were plated in 60-mm dishes at a density of 6×10^5 cells/dish and grown in DMEM hi-glucose for 24 h prior to DNA transfection using LipofectAMINE 2000 reagent. Treatment with DHT and subsequent incubation times paralleled the 96-well plate protocols described above and in the legend of Figure 1. Cells were then harvested in 100 μl of RIPA buffer (10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.2 mM Na_3VO_4, pH 7.2) that contained a cocktail of mammalian protease inhibitors (Sigma-Aldrich). Equal volumes of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and probed with anti-AR (N20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAL4-DBD monoclonal antibody (Clontech), anti-VP16-AD polyclonal antibody (Clontech) and anti-actin antibody (Santa Cruz Biotechnology). HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and goat-anti mouse HRP-conjugated antibody (BD Biosciences, San Jose, CA, USA) were used as secondary antibodies. Detection was carried out using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and an enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturers' protocols. Coomassie Brilliant Blue staining of the gel showed no significant differences in protein loading. Chemiluminescent images were analyzed by a Fluor-S Max Multilmager Quantification System (Bio-Rad, Hercules, CA, USA).

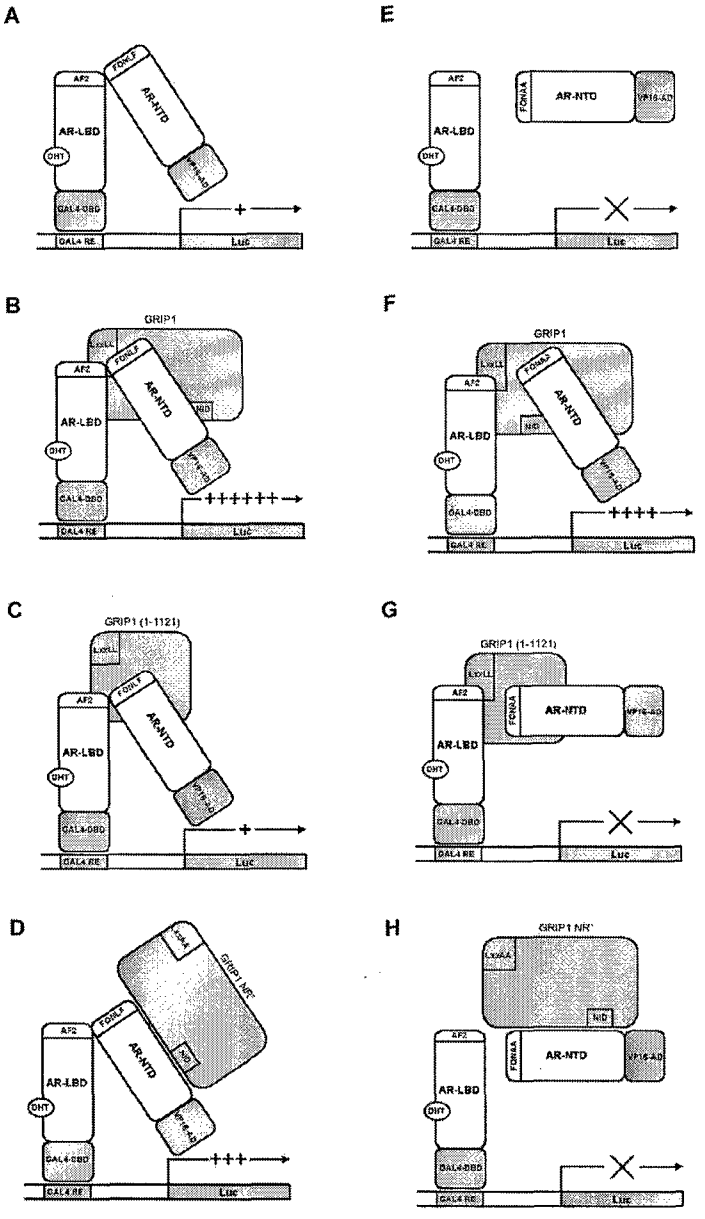

Figure 3 Molecular interactions involved in the AR N/C mammalian two-hybrid assay.
The DHT-induced VP16 AR-NTD/GAL4 AR-LBD interactions driving expression of luciferase reporter in the absence or presence of GRIP1 coactivator are depicted schematically. The relative strength of luciferase activity detected is represented symbolically using plus symbols (+) to indicate activity detected above background levels, or crosshatch (X) indicating little to no luciferase activity detected. All models depict scenarios following DHT binding to AR-LBD. Models A, B, C, and D depict actions of wild-type AR-NTD ^{23}FQNLF^{27} in the assay. Models E, F, G, and H depict actions of mutant AR-NTD ^{23}FQNAA^{27} in the assay. (A,E) AR-LBD interacts through the AF2 motif with the ^{23}FQNLF^{27} motif of wild-type AR-NTD. This interaction is lost by mutant AR-NTD ^{23}FQNAA^{27}. (B,F) The presence of wild-type GRIP1 enhances reporter activity through bridging of AR N/C interaction. Bridging is mediated by GRIP1 LXXLL and NID_{AF1} motifs binding to respective domains of AR. GRIP1 bridging results in rescue of the impaired AR-NTD ^{23}FQNAA^{27}/AR-LBD interaction. (C,G) Truncated GRIP1 (1-1121) fails to bind AR-NTD, cannot bridge across AR domains, and thus has no impact on DHT-induced AR N/C interaction. (D,H) Mutant GRIP1 NR* fails to bind AR-LBD. Marginal GRIP1 NR* recruitment to the transcription complex may occur through preserved binding to AR-NTD, resulting in modest enhancement of reporter activity in the context of wild-type AR N/C interaction.

In summary, we have demonstrated that GRIP1 acts as a bridge between AR-NTD and -LBD. Furthermore, a disparity between AR N/C interaction and AR transactivation activity was evident, since the two processes had different DHT dependence and GRIP1 mutants were unable to rescue the impaired AR N/C interaction experienced by an AR-NTD mutant, while the transactivation activity of this AR mutant remained largely intact. We therefore conclude that AR N/C interaction, as mediated either directly by FQNLF/AF2 interaction or indirectly by GRIP1 bridging, is involved in the enhancement of, but is not necessary for, AR transactivation activity. The data

presented here indicate that the relatively large and unstructured AR-NTD is pleiotropic in its transcriptional regulation, and strongly suggest that this flexibility may be utilized in response to changing cellular signaling environments during normal cell growth and differentiation, as well as abnormal situations such as during the progression of prostate cancer.

Acknowledgments

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Androgen Receptor–Dependent PSA Expression in Androgen-Independent Prostate Cancer Cells Does Not Involve Androgen Receptor Occupancy of the *PSA* Locus

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Abstract

It is widely suspected that androgen-independent prostate cancer growth depends on androgen receptor signaling via ill-defined mechanisms. Prostate-specific antigen (PSA) expression is often used to measure androgen receptor activity in cells and prostate cancer progression in patients. In the present study, we have compared androgen receptor activity using PSA and human male germ cell–associated kinase (hMAK), as read-outs in androgen-dependent LNCaP and androgen-independent C4-2B cells. As expected, very little PSA and hMAK expression were detected in LNCaP cells in the absence of androgens, whereas substantial expression of PSA was observed only in C4-2B cells under the same conditions. The addition of dihydrotestosterone to the culture medium increased the expression of both genes in both cell types. Comprehensive chromatin immunoprecipitation analysis of the entire *PSA* locus and an androgen-response element in *hMAK* unexpectedly revealed that androgen receptor was not occupying any site in the absence of dihydrotestosterone in either cell type. In line with the expression data, and in the absence of dihydrotestosterone, histone acetylation and RNA polymerase II occupancy was substantial at the *PSA* locus in C4-2B but not in LNCaP cells. In the presence of dihydrotestosterone, androgen receptor was found to occupy mainly the enhancer region of *PSA* in both cell types, accompanied with increases in histone acetylation and RNA polymerase II occupancy. Although the androgen receptor was not directly involved in the androgen-independent expression of PSA in C4-2B cells, small interfering RNA knock-down of androgen receptor significantly reduced PSA expression in both the presence and absence of dihydrotestosterone. In contrast, hMAK expression was decreased only in the presence of dihydrotestosterone after androgen receptor knock-down. We conclude that androgen-independent expression of PSA in C4-2B cells does not rely on the direct occupancy of the androgen receptor at the *PSA* locus, but is nevertheless affected indirectly via unknown androgen receptor–dependent mechanism(s) that influence the expression from some but not all androgen receptor target genes. (Cancer Res 2005; 65(17): 8003-8)

Introduction

Androgen ablation therapy of prostate cancer, although initially efficacious, nearly always leads to treatment resistance and progression of the disease (1). Although the disease is then referred to as androgen-independent, it simply reflects resistance to the original therapy protocol. Mechanisms underlying androgen independence are thought to involve an adaptation of androgen receptor signaling to function under low or absent androgen levels (2). Prostate-specific antigen (*PSA*) is an androgen-responsive gene and its expression is often used as a measure of androgen receptor signaling and as a monitor of prostate cancer progression (3). Human male germ cell–associated kinase (*hMAK*) is a newly identified target gene of the androgen receptor that may participate in prostate cancer cell physiology (4). Because the role of the androgen receptor in androgen-independent prostate cancer is not clearly defined, we have embarked on a detailed analysis of androgen receptor signaling measuring PSA and hMAK expression in LNCaP and C4-2B cells. LNCaP cells were originally obtained from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate cancer (5). The cells are responsive to dihydrotestosterone in terms of growth and PSA expression. Although the androgen receptor in LNCaP cells is mutated (6), it is generally normal with respect to transactivation activities (7). C4-2B is a subline of LNCaP obtained by passage and growth in castrated athymic mice (8). The cells are no longer responsive to hormone manipulation in animals (although they still express a functional androgen receptor; ref. 9) and metastasize readily to lymph nodes and bone in athymic mice. Our results reveal the striking finding that androgen receptor–dependent PSA expression in C4-2B cells does not involve androgen receptor occupancy at the *PSA* locus.

Materials and Methods

Cell culture and materials. Human prostate cancer LNCaP cells, obtained from the American Type Culture Collection (Manassas, VA) and C4-2B, obtained from ViroMed Laboratories (Minneapolis, MN), were both maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum. 5α-Dihydrotestosterone was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were anti–androgen receptor NH_2-terminal (N20), anti–androgen receptor COOH-terminal (C19) anti-Pol II (N20), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AcH3 (Upstate Biotechnology, Inc., Lake Placid, NY) and anti-PSA (DAKO Corp., Carpinteria, CA). Immunoblotting was done as described previously (10), with the indicated antibodies.

Real-time reverse transcription-PCR. After the indicated treatments of LNCaP and C4-2B cells, total cellular RNA was prepared using SV Total RNA Isolation System (Promega, Madison, WI). A two-step reverse transcription-PCR method was employed using the TaqMan Gold RT-PCR kit (Applied Biosystems, Branchburg, NJ) and primers and probes for *PSA* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression were as previously described (10). Primers and probe for *hMAK* expression were: 5′-GGTCACAGCCACCATACACTGA-3′ (forward); 5′-ACTTCCAACAGCCCACA-CATC-3′ (reverse); 5′6-FAM-CCAGATGGTATCGTGCCCCTGAAGTTT-BHQ-1-3′ (probe). Triplicate PCR reactions were done. *GAPDH* mRNA expression was analyzed for each sample in parallel. The results are represented as *PSA* or *hMAK/GAPDH* mRNA ratios.

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Transient transfection and luciferase assays. LNCaP (6×10^5 cells/well) and C4-2B (3×10^5 cells/well) were plated in six-well plates and grown in phenol red–free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum (CSS) for 2 days. Cells were then transfected with an androgen receptor–responsive reporter, pGL3-PSA5.85 (2 μg/well) using LipofectAMINE 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. PGL3-PSA5.85 (a gift from Dr. Hong-Wu Cheng) was constructed by inserting the entire 5.85 kb *PSA* upstream sequence into pGL3 basic vector (11). After transfection, cells were grown in phenol red–free RPMI 1640 containing 5% CSS with dihydrotestosterone or vehicle control for 24 hours. Luciferase activity determinations in cell lysates were conducted as previously described (10).

Chromatin immunoprecipitation assays. LNCaP (6×10^6 cells/150 mm dish) and C4-2B (3×10^6 cells/150 mm dish) were cultured in phenol red–free RPMI 1640 supplemented with 5% CSS for 3 days. Cells were treated with dihydrotestosterone or vehicle control for 4 hours, and chromatin immunoprecipitation (ChIP) assays were conducted as described previously (10). DNA samples from ChIP preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad, Hercules, CA) and AmpliTaq Gold PCR master mix (Applied Biosystems). The primers and probes (synthesized by Biosearch Technologies, Novata, CA) were (as they relate to the positions at the *PSA* locus; see Fig. 2):

A 5′-GCCTGGATCTGAGAGAGATATCATC-3′ (forward)
5′-ACACCTTT-TTTTTTCTGGATTGTTG-3′ (reverse)
5′-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3′ (probe)

B 5′-CTGGGCAAGCACAATCTGA-3′ (forward)
5′-GTGTGATGCTGGATGATGAGTG-3′ (reverse)
5′-6-FAM-CATGAAAGATGCCCCAGAGGCCTTG-BHQ-1-3′ (probe)

C 5′-CAGTGGCCATGAGTTTTGTTTG-3′ (forward)
5′-AACCAATCCAACTGCATTATACACA-3′(reverse)
5′-6-FAM-CCCAACG-CAACTTAACCTAACAAG-BHQ-1-3′ (probe)

D 5′-TTCCCAGGCTGGAGTTCAATAG-3′ (forward)
5′-GGAGACCGAAGCAGGAGAATC-3′ (reverse)
5′-6-FAM-CAGCTCACTGTAGCCTTGAACTCCTGGC-BHQ-1-3′ (probe)

E 5′-CCTAGATGAAGTCTCCATGAGCTACA-3′ (forward)
5′-GGGAGGGAGA-GCTAGCACTTG-3′ (reverse)
5′-6-FAM-CAATTACTAGATCACCCTGGATGCACCAGG-BHQ-1-3′ (probe)

F 5′-CACACCCGCTCTACGATATGAG-3′ (forward)
5′-GAGCTCGGCAGGCTCTGA-3′ (reverse)
5′-6-FAM-CTCCAGCCACGACCTCATGCTGCT-BHQ-1-3′ (probe)

G 5′-TCATCATGAATCGCACTGTTAGC-3′ (forward)
5′-GCCCAAGTGCCTTGGTATACC-3′ (reverse)
5′-6-FAM-TGAATCATCTGGCACGGCCCAA-BHQ-1-3′ (probe)

The primers and probes (synthesized by Biosearch Technologies) were (as they relate to the indicated *hMAK* locus; see Fig. 4):

- 5′-AGAAGTTGGAGACGACCCTGAA-3′ (forward)
- 5′-CAACTAGAGAATCAACGATGGAGTCA-3′ (reverse)
- 5′-6-FAM-CCAACAACATCAAGCCAAATGCAACTATTG-BHQ-1-3′ (probe).

Duplicate immunoprecipitations for each antibody and triplicate PCR reactions for each immunoprecipitation sample were done. The results are given as a percentage of input.

Small interfering RNA transfection. C4-2B cells (1.5×10^5 cells/well) were plated in six-well plates and grown in phenol red–free RPMI 1640 containing 5% CSS for 2 days. Cells were transfected with one of two androgen receptor small interfering RNA (siRNA) duplexes. The first was sense, 5′-AUG UCA ACU CCA GGA UGC UTT-3′; and antisense, 5′-AGC AUC CUG GAG UUG ACA UTT-3′, directed against the coding region of androgen receptor mRNA. The second was sense, 5′-ACG UUU ACU UAU CUU AUG CTT-3′; and antisense, 5′-GCA UAA GAU AAG UAA ACG UTT-3′, directed against the 3′-untranslated region of androgen receptor mRNA. A nonspecific siRNA duplex, 5′-AGA UCU GGC UAU CGC GGU ATT-3′ and 5′-UAC CGC GAU AGC CAG AUC UTT-3′ was used as a control. All siRNA duplexes were at a final concentration of 100 nmol/L and transfected using OligofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown in phenol red–free RPMI 1640 containing 5% CSS for 48 hours and then treated with dihydrotestosterone or vehicle control for 18 hours. Total RNA and protein extractions were conducted for reverse transcription-PCR and immunoblot analyses, respectively, as described above.

Results

PSA and hMAK expression in LNCaP and C4-2B cells. In LNCaP cells, virtually no detectable PSA expression was observed in the absence of androgens as measured by immunoblot of the PSA protein (Fig. 1*A*) or expression of *PSA* mRNA (Fig. 1*B* and *C*). On the other hand, PSA expression was substantial in C4-2B cells in the absence of androgens (Fig. 1*A-C*). In contrast, the low level of hMAK expression in the absence of dihydrotestosterone stimulation was not higher in C4-2B cells compared with LNCaP (Fig. 1*D*). Addition of dihydrotestosterone increased the expression of PSA and hMAK in both cell lines; expression values of PSA in C4-2B cells in the absence of dihydrotestosterone were similar or even higher than values in LNCaP cells in the presence of androgens. Even at low concentrations of dihydrotestosterone, C4-2B expressed more than an order of magnitude higher *PSA* mRNA than did LNCaP cells at any given dihydrotestosterone concentration (Fig. 1*C*). However, when a transiently-transfected PSA-driven luciferase reporter was used, virtually no detectable luciferase expression was measured in the absence of androgens in either LNCaP cells or C4-2B cells (Fig. 1*E*). Therefore, the observed ligand-independent expression of endogenous PSA in C4-2B cells is somehow related to the chromatin-integrated nature of the native *PSA* gene (see below).

Chromatin immunoprecipitation analysis of the *PSA* and *hMAK* loci. In previous work, we began to define transcriptional activity at the *PSA* locus in terms of androgen receptor and cofactor occupancies and histone modifications (7, 10, 12). We have now extended the analysis to interrogate seven sites at the locus spanning the enhancer, promoter, an exon and 3′-untranslated region of the gene (Fig. 2*A*). Androgen receptor recruitment, histone H3 acetylation and RNA polymerase II occupancy were analyzed in LNCaP and C4-2B cells in the absence or presence of dihydrotestosterone stimulation (Fig. 2*B*). The IgG-negative controls gave very low signals throughout in both cell lines. No androgen receptor occupancy was detected in either cell line in the absence of dihydrotestosterone using an NH_2-terminal androgen receptor antibody (N20; Fig. 2*B*) or a COOH-terminal antibody (C19; Fig. 3), whereas significant occupancy was observed at the enhancer (A) and to a lesser extent at the promoter (E) in both cell lines in the presence of dihydrotestosterone (Figs. 2*B* and 3). This is an interesting finding considering the high expression levels of PSA in C4-2B cells in the absence and presence of dihydrotestosterone (orders of magnitude higher than in LNCaP cells), yet no androgen receptor was found at the *PSA* locus in the absence of dihydrotestosterone, and in the presence of dihydrotestosterone, androgen receptor occupancy was lower in C4-2B than in LNCaP cells. The fact that this result was obtained using androgen receptor antibodies directed to two different epitopes indicated that it is unlikely that epitope masking

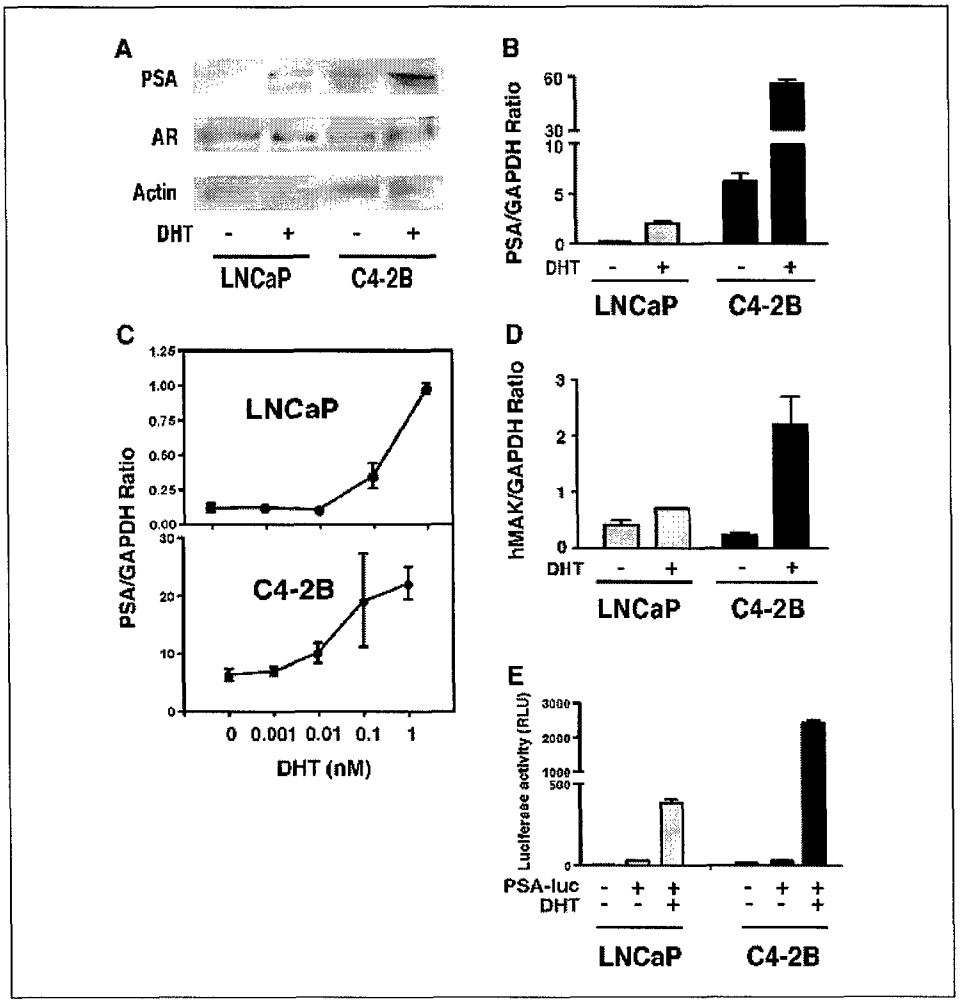

Figure 1. PSA expression in LNCaP cells and C4-2B cells. *A*, LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with dihydrotestosterone (10 nmol/L) or vehicle control (ethanol) for 18 hours. Immunoblots were conducted using anti-PSA, anti–androgen receptor and anti-actin antibodies. *B*, LNCaP cells and C4-2B cells were treated as in (*A*). *PSA* and *GAPDH* mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as *PSA/GAPDH* mRNA ratios. Values are presented as means ± SD of triplicate real-time PCR assays. *C*, LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with different concentrations of dihydrotestosterone as indicated for 18 hours. *PSA* and *GAPDH* mRNA levels were measured as in (*B*). *D*, cDNA obtained from (*B*), was analyzed for *hMAK* mRNA levels. *E*, LNCaP and C4-2B cells were transiently transfected with pGL3-PSA5.85 and incubated with dihydrotestosterone (10 nmol/L) or vehicle control for 24 hours. Values are presented as the means ± SD of duplicate wells. Triplicate samples of each well was read in a luminometer, and the average was normalized to total protein concentration. RLU, relative luciferase units.

is the reason for the lack of androgen receptor detection at these loci in the absence of dihydrotestosterone treatment. The results, therefore, indicate that a significant proportion of PSA expression occurs either via non–androgen receptor mechanisms or androgen receptor–dependent mechanisms not related to androgen receptor occupancy at the *PSA* locus in C4-2B cells. Histone H3 acetylation and RNA polymerase II occupancy was substantially higher in C4-2B than LNCaP cells in the absence of dihydrotestosterone (at A, B, E, and F sites) in line with dihydrotestosterone-independent expression of PSA in these cells. This indicates that these regions, especially E and F, are in readily accessible chromatin, which likely facilitate the recruitment of general basal transcription factors and RNA polymerase II to mediate the observed transcription of *PSA* mRNA. Dihydrotestosterone addition resulted in a further increase of these variables in C4-2B cells, which was now also detected in LNCaP cells. Overall, these results indicate that PSA expression in the absence of dihydrotestosterone occurs in C4-2B cells in the absence of androgen receptor occupancy at this locus.

The same ChIP DNA obtained for the experiment depicted in Fig. 2 was assessed for an androgen-response element located about 3.5 kb upstream from the transcription start site of the *hMAK* gene (Fig. 4).

The androgen-response element (GGAACATGATGGCCT) was identified by scanning the 5′-upstream region of the *hMAK* gene using ConSite (web-based prediction of regulatory elements; ref. 13). In the absence of dihydrotestosterone, very low to negligible androgen receptor occupancy, histone acetylation or polymerase II occupancy were detected at this site in both cell lines consistent with the very low expression levels of this gene under these conditions. Note that this result is in contrast with the observations at the *PSA* locus in C4-2B cells. The addition of dihydrotestosterone to the medium resulted in increases in all the parameters, especially in C4-2B cells, again consistent with expression values obtained for this gene under these conditions. The only inconsistency noted between ChIP results and expression levels of the two genes is the lack of androgen receptor occupancy at the *PSA* enhancer in the absence of dihydrotestosterone in C4-2B cells, whereas substantial expression of PSA occurred under these conditions in the cells. We therefore tested whether the androgen receptor is even involved under these conditions.

Androgen receptor involvement in PSA and hMAK expression levels in the absence of dihydrotestosterone in C42B cells. To determine whether the androgen receptor was involved, even indirectly, in PSA expression in the absence of dihydrotestosterone in C4-2B cells, we knocked down the androgen receptor using a siRNA approach and measured *PSA* mRNA expression (Fig. 5). As a negative control, we also assayed the expression of *hMAK* using the same cDNA preparation, and as a positive control, the expression of these genes when the cells were stimulated by dihydrotestosterone. In the experiment depicted, we used the siRNA directed against the coding region of androgen receptor mRNA (see

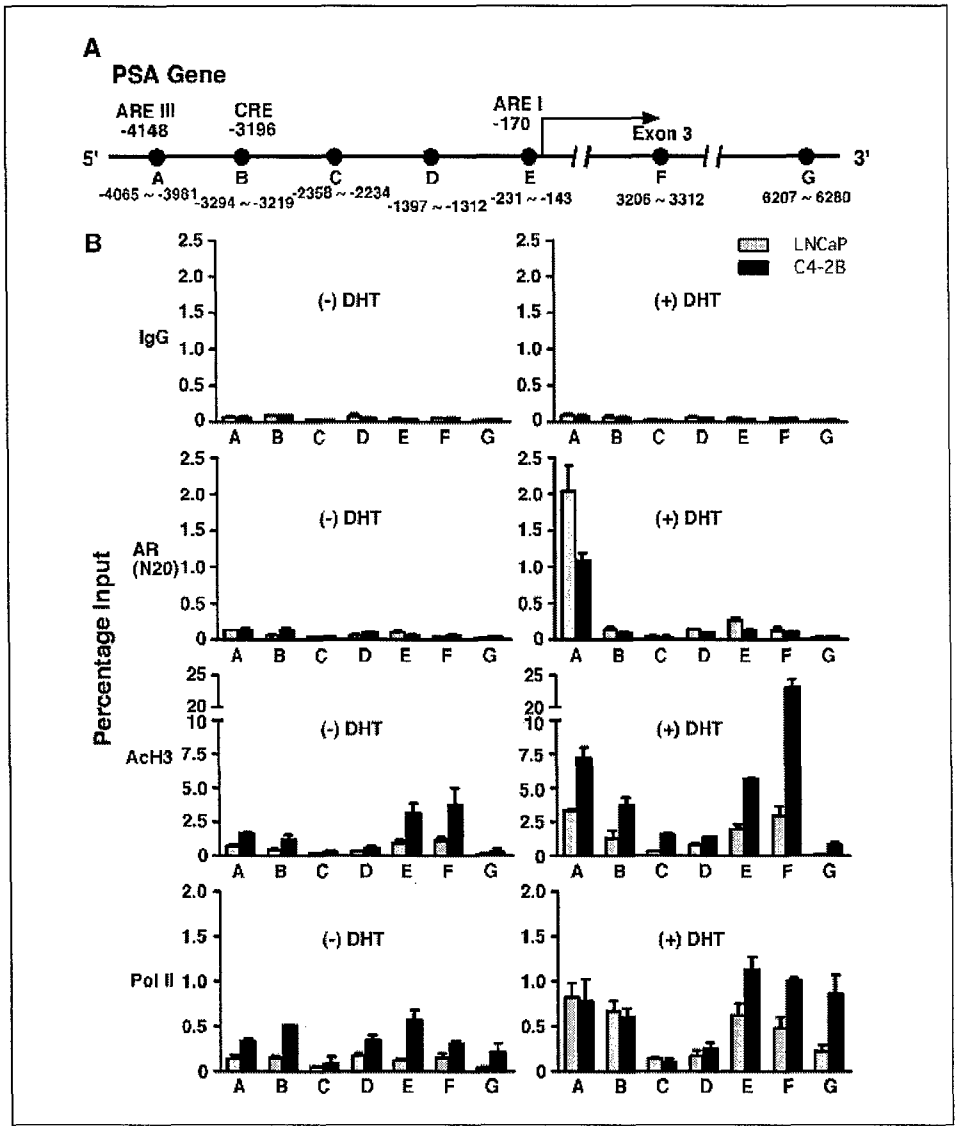

Figure 2. ChIP analysis at *PSA* locus. *A*, schematic representation of the *PSA* gene. ARE, androgen response element; CRE, cyclic AMP–responsive element; ●, real-time PCR targeted regions; arrow, transcription start site. *B*, LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancy (using N20 antibody), histone H3 acetylation and RNA Pol II recruitment were examined by ChIP analyses. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations; columns, mean; bars, ± SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

Materials and Methods). Androgen receptor was knocked-down successfully as revealed by immunoblot analysis (Fig. 5*A*). This maneuver caused a substantial inhibition of both androgen-dependent and -independent PSA expression roughly to the same extent as the androgen receptor protein was knocked down (Fig. 5*B*). Similar results were obtained using a siRNA directed against the androgen receptor mRNA 3′-untranslated region (data not shown), indicating that the results were not due to off-target effects

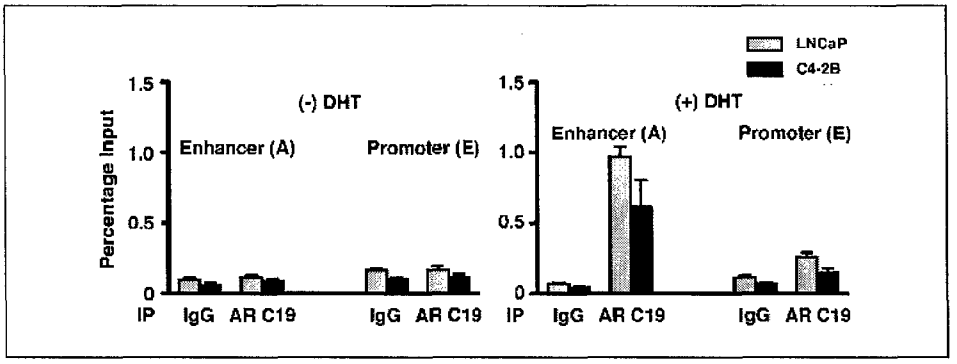

Figure 3. Androgen receptor ChIP with COOH-terminal androgen receptor antibody. LNCaP and C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 3 days and then treated with or without dihydrotestosterone (10 nmol/L) for 4 hours. Androgen receptor occupancies on *PSA* enhancer and promoter were examined by ChIP analyses using the antibody against androgen receptor COOH terminus. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations, and are presented as percentage input and represent mean values ± SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

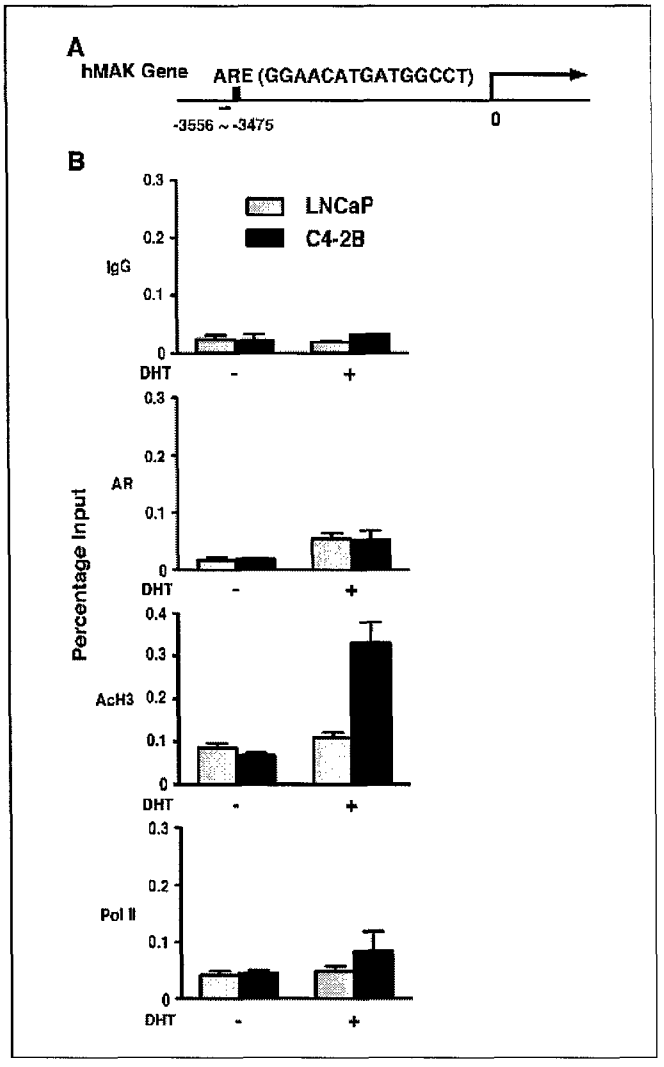

Figure 4. ChIP analysis at *hMAK* locus. *A*, schematic representation of the *hMAK* gene. ARE, androgen response element; arrow, transcription start site. *B*, ChIP DNA samples obtained from the experiment depicted in Fig. 2*B* were analyzed for androgen receptor occupancy, histone H3 acetylation and RNA Pol II recruitment at the *hMAK* locus. The value of each immunoprecipitation sample was obtained from the average of triplicate real-time PCR assays from duplicate immunoprecipitations, and values are presented as percentage input and represent mean values ± SD. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were done.

of the siRNA. Using the same cDNA, no equivalent decrease in the low but measurable level of *hMAK* expression was observed (Fig. 5*C*). The decrease in dihydrotestosterone-stimulated activity observed with the nonspecific siRNA treatment might be related to a general siRNA effect or possibly due to stimulation of the IFN system elicited by double-stranded RNA (14). This was not seen in the PSA expression activity in the absence of dihydrotestosterone. The fact that androgen receptor knock-down caused PSA expression inhibition (but not hMAK expression inhibition) in the absence of dihydrotestosterone, indicates that the androgen receptor is somehow involved in PSA expression specifically. The involvement, however, seems to be indirect because no androgen receptor occupancy at the androgen receptor enhancer was apparent in our ChIP analyses in the absence of dihydrotestosterone treatment in C4-2B cells (Figs. 2 and 3).

Discussion

The androgen receptor signaling axis is involved in all phases of prostate cancer, from genetic predisposition through disease progression, including the development of resistance to androgen

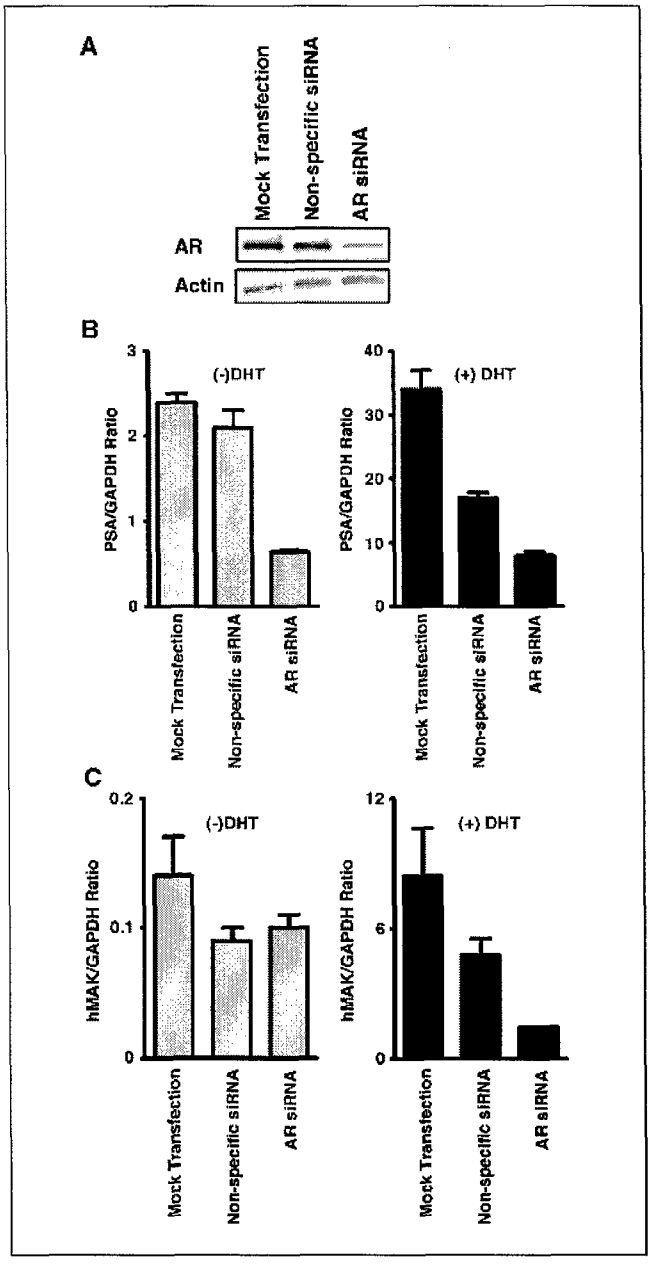

Figure 5. PSA and hMAK expression in C4-2B cells after siRNA-mediated knock-down of androgen receptor. *A*, C4-2B cells were incubated in phenol red–free RPMI 1640 containing 5% CSS for 2 days and then mock-transfected or transfected with androgen receptor siRNA (100 nmol/L) directed against the coding region of the androgen receptor mRNA or the same amount of nonspecific siRNA. After 48 hours, androgen receptor protein levels were measured by immunoblot analysis. The membrane was stripped and reprobed with an anti-actin antibody. *B* and *C*, 48 hours after androgen receptor siRNA or nonspecific siRNA transfection, C4-2B cells were treated with or without dihydrotestosterone (10 nmol/L) for 18 hours. *PSA* (*B*) and *hMAK* (*C*) mRNA levels were determined by real-time reverse transcription-PCR. The PSA expression values are shown as PSA or *hMAK/GAPDH* mRNA ratios. Columns, mean; bars, ± SD. Results shown are representative of three independent experiments. Similar results were obtained when a siRNA directed against the androgen receptor mRNA 3′-untranslated region was used.

ablation therapies. Resistance to androgen ablation is not due to a loss of androgen responsiveness, but in most cases, is associated with altered androgen receptor activity (reviewed in refs. 2, 15, 16). Such aberrant androgen receptor signaling is thought to include androgen receptor hypersensitivity to androgens, androgen receptor insensitivity to antagonists, increased androgen receptor expression levels, or androgen receptor activity modulation by nonsteroidal signaling pathways. Although the details of these molecular processes are largely unknown, they may be linked, operating together in a given tumor. It was recently shown that increased androgen receptor expression levels are necessary and sufficient to convert prostate cancer to an ablation-resistant state (17).

The main finding of the present work, which adds another dimension to the notions referred to above, is that the androgen receptor is involved in PSA expression in the absence of androgen in the androgen-independent cell line, C4-2B, without occupying regulatory sites in the *PSA* upstream control region. What are the possible mechanisms to explain this? This question is not trivial because as stated above, androgen-independent receptor activity may be one of the main mechanisms governing growth in androgen-independent, advanced prostate cancer.

One possibility to explain our findings is that the androgen receptor may affect the transcription of other transcription factors that in turn activate PSA expression. Candidates for such indirect effects are general transcription factors like SP1 or transcription factors affected by non–steroid hormone signals like cyclic AMP–responsive element binding protein, for which response elements exist at the *PSA* locus. We recently analyzed the involvement of cyclic AMP–responsive element binding protein at the *PSA* locus, and although we could define an enhanceosome with cooperation between the androgen receptor and cyclic AMP–responsive element binding protein via p300/CBP, its contribution to overall transcription was relatively small and could only be observed by protein kinase A stimulation with forskolin (18). This does not exclude the involvement of other transcriptional factors in the androgen-independent activation of PSA expression with which the androgen receptor may cooperate in an indirect fashion.

Another and even more intriguing mechanism of androgen receptor–dependent, but androgen-independent PSA expression, may be related to the nongenomic actions of the androgen receptor as proposed to be active in many mammalian cell types (19). Such nonclassical androgen receptor mechanisms may work via classic nonsteroidal signaling pathways affecting any number of other transcription factors. For example, it was reported that androgen receptor forms a triple complex containing androgen receptor/p85α/Src affecting phosphoinositide-3-kinase/Akt activation and thus mediating androgen-induced cell growth and survival (20). More recently, it was reported that the Src-MEK-1/2-ERK-1/2-cyclic AMP–responsive element binding protein pathway, associated with the transition of LNCaP cells to androgen-independence, correlates with the nongenotropic actions of androgen receptor where nuclear translocation and the ability of the androgen receptor to bind to DNA were not prerequisites for androgen receptor activity (21). The challenge therefore will be to identify and understand the actions of such factors and signaling mechanisms not only to better understand the progression of androgen-independent prostate cancer, but also to devise schemes of better therapies for this life-threatening disease.

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Mini review

Molecular chaperones throughout the life cycle of the androgen receptor☆

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Abstract

Aberrant signaling by the androgen receptor contributes to the initiation and progression of prostate cancer. The involvement of molecular chaperones in the processes of folding, activation, trafficking, and transcriptional activity of the androgen receptor provide different points along the signaling axis where regulation of androgen receptor activity can be hijacked to provide growth signals for clonal selection in cancer progression. Evidence exists of abnormal chaperone expression that could contribute to the upregulation of AR activity in prostate tumors. Regardless of whether chaperones are involved in the causation of prostate carcinogenesis, molecular chaperones provide therapeutic targets for the treatment of prostate cancer.

Keywords: Androgen receptor; Chaperones; Nuclear translocation; Transcription

1. Introduction

The androgen receptor (AR) is a transcription factor belonging to the class I subgroup of the nuclear receptor superfamily. Members of this superfamily are ligand-responsive and share structural and functional similarities with one another [1]. Stimulated by androgens, the AR signaling pathway plays an important role in the development and differentiation of target tissues [1,2], as well as a critical role in the initiation, proliferation, and progression of prostate cancer to ligand-independent disease [3,4].

The predominantly cytoplasmic AR rapidly translocates into the nucleus in response to androgen [5,6]. As with any signal transduction pathway, proper signaling is dependent upon the receptor's ability to acquire more than one conformational state [7]. Unbound to ligand, the AR is maintained in an inactive, but highly responsive state by a large dynamic heterocomplex composed of heat shock proteins, co-chaperones, and tetratricopeptide repeat (TPR)-containing proteins [8]. Most of the androgen receptor-chaperone interactions identified so far appear to involve the ligand-binding domain (LBD)

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of the receptor. Deletion of the AR-LBD abolishes hormone-responsiveness, and results in the constitutive activity of the receptor [2,5]. Therefore, neither the LBD nor hormone is believed to be necessary for AR transactivation activity. Instead, the primary function of the LBD may be to inhibit activation of the AR and, therefore, the transcriptional activation of its target genes in the absence of hormone. Binding of 5α-dihydrotestosterone (DHT) to AR relieves the inhibition imposed by the LBD [2].

The molecular chaperone complex is believed to configure the ligand-binding domain into a relatively stable, partially unfolded, inactive intermediate with a high-affinity for DHT [7,8]. Hormone binding to the complex permits the folding of the AR into an active conformation [2,8]. Binding of ligand, followed by the dissociation of the receptor-chaperone complex and activation of the receptor is viewed as the general regulatory mechanism of AR signaling [9]. However, molecular chaperones remain important players in the events downstream of receptor activation, and throughout the life cycle of the AR (see below).

2. Minimal molecular chaperone complex (Fig. 1)

Initially identified by their accumulation in response to cellular stress, chaperone proteins are able to recognize and bind to hydrophobic regions on unfolded or partially folded proteins preventing their irreversible aggregation, promoting cycles of chaperone-mediated folding instead [10,11]. However, even under normal cellular conditions molecular chaperones assist in the proper folding of steroid hormone receptors. The structural conformation of the LBD required for AR activation is accomplished through multiple cycles of binding and release of the AR by the components of the multichaperone complex [7,10,12]. Without these chaperone proteins, the receptor is not denatured. Instead the receptor acquires a conformation where the hydrophobic pocket within the ligand-binding domain remains inaccessible to hormone, preventing activation. Therefore, the receptor is required to be in a partially unfolded high-affinity ligand-binding conformation stabilized by the chaperone heterocomplex in order to be responsive to stimuli [13].

Much of the work on the chaperone-steroid hormone relationship prior to the 1990s did not specifically focus on the AR. Prior studies had identified receptor-Hsp90 heterocomplexes for the glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor (MR), and estrogen receptor (ER), which sediment at ~9S [8]. The highly conserved LBD among nuclear receptors [14] and the existence of a ~9S form of AR [8] suggested that a similar AR-Hsp90 heterocomplex existed as well. Later studies confirmed that Hsp90 does in fact interact with the LBD of the AR along with Hsp70 and Hsp56/FKBP52 [15,16], which were required to maintain the AR in a high affinity ligand-binding state that is hormone- [17] and temperature-dependent [18,19].

The basic heterocomplex, dubbed the 'foldosome', generally required for the efficient folding and stabilization of steroid hormone receptors consists of Hsp70 (hsc70), Hsp40 (Ydj1), Hop (p60), Hsp90, and p23 [20,21]. This minimal complex is believed to be essential for ligand responsive signaling of all full-length steroid hormone receptors [7]. Subcomplexes of chaperone proteins lacking receptor or other substrate peptides have been found to exist in the cytosol [22]. However, the assembly of steroid hormone receptors into the ligand competent state is believed to occur in an assembly-line type of process [8,23,24]. Having been shown to associate with ribosomes during translation [25], the first chaperone protein likely to interact cotranslationally with the AR is Hsp70 along with its co-chaperone Ydj1, a DnaJ-like member of the Hsp40 family. Ydj1 augments Hsp70's ability to reversibly bind to small stretches of hydrophobic amino acids [10,12,26] on the nascent peptide to maintain the receptor in a soluble state, preventing irreversible aggregation [27]. Ydj1 may actually bind to the non-native peptide prior to Hsp70 binding to facilitate the transfer of AR to Hsp70 [28]. Ydj1 is hypothesized to potentially assist in correctly aligning the Hsp70 and receptor interaction surfaces [11].

After the initial binding of Hsp70 and Ydj1 to the receptor, Hip/p48 (hsc70 interacting protein), another Hsp70 co-chaperone, binds to stabilize and prolong the interaction between the receptor and this intermediate chaperone complex [29]. Hop (Hsp organizer protein) then binds to the complex and recruits a homodimer of Hsp90 [30] by physically forming a bridge between Hsp70 and Hsp90 using its TPR motifs in the interactions. In addition, Hop contributes

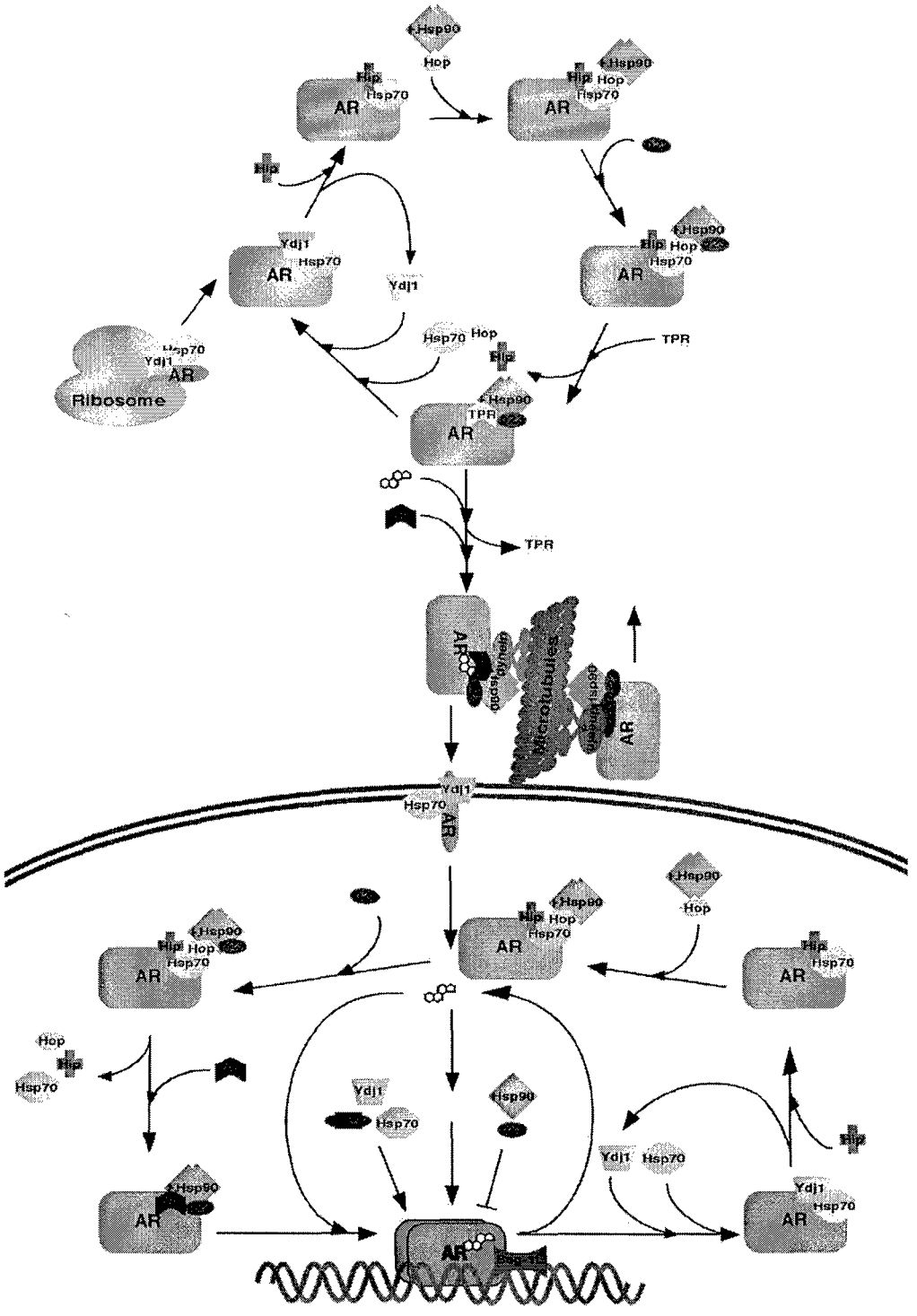

Fig. 1. The itinerary of the AR with emphasis on the role of molecular chaperones.

to more efficient folding by influencing conformational changes within Hsp70 and Hsp90 [13]. To stabilize the AR in the high-affinity, inactive intermediate conformation, p23, a small molecular weight chaperone, binds to Hsp90 within the complex as Hip, Hop, and Hsp70 dissociate [30–33]. The release of Hop allows for the interaction of a TPR-containing immunophilin with Hsp90, generating the 'final complex' [9].

The interactions between steroid hormone receptors and molecular chaperones are transient [19,34] resulting in a dynamic folding/refolding cycle [13] driven by conformational changes within Hsp90 [32]. In the absence of hormone, an equilibrium is established which maintains the AR in an overall high-affinity ligand-binding state. Hormone binding causes the receptor to undergo a sequential loss of chaperones, a process thought to occur as a result of blocking the formation of the receptor-Hsp70 complex and, therefore, the subsequent interactions that occur in the assembly/disassembly cycle of the disactivation loop [19]. With the assistance of Hsp90 [32], transformation of the receptor into the DNA-binding competent state [16] is followed by nuclear translocation, recruitment of cofactors, and transcription of target genes [32].

3. AR Translocation (Fig. 1)

Based on studies of PR, ER, and GR, it is known that steroid hormone receptors undergo continuous nucleocytoplasmic shuttling [35] with the predominant localization determined by the accessibility of the nuclear localization signal (NLS) [36]. The NLS of the AR, assumed to be hidden in the inactive conformation, is unmasked upon receptor activation allowing transport into the nucleus [37]. Nuclear transfer of the AR has been shown to be hormone concentration- [6] and Hsp90-dependent [32].

A recent study proposes that the earliest step in receptor activation is not the dissociation of the heterochaperone complex, but the exchange of one TPR-containing immunophilin for another along with the recruitment of cytoplasmic dynein [31]. This, in conjunction with the reported finding of an Hsp90 interaction with the cytoskeleton [12], provides a mechanism for the translocation and accumulation of the AR within nuclear foci [37,38]. The model developed proposes the involvement of Hsp90 complexes in the trafficking of the receptor along microtubules, using the particular TPR-containing protein associated with the heterocomplex as a guide to sites of receptor action [39]. Once the receptor is activated, the association of an immunophilin with the Hsp90 complex is thought to guide the receptor in a retrograde direction along microtubules. Whereas, binding of Cdc37 (p50) is believed to be the Hsp90-associated protein that is responsible for anterograde trafficking [13,36].

Hsp70 proteins have also been found to associate with steroid hormone receptors in the presence of ligand [40] and are thought to play a role in translocating the receptor across intranuclear membranes presumably by temporarily unfolding the receptor [12,41]. Ydj1, a putative component of the matrix lamina pore complex, may assist Hsp70 in unfolding/refolding the AR as the receptor is transported across the nuclear membrane [42]. Once inside the nucleus, the chaperone complex dissociates from the receptor [31].

4. Transcriptional activation

In addition to the influence of cofactors on AR activity, there is also evidence for molecular chaperone involvement in the activation of target genes. Bag-1 proteins, which are Hsp70 co-chaperones, are involved in hormone receptor transactivation. Specifically Bag-1L, the long, constitutively nuclear isoform of the Bag-1 proteins [43] has been shown to complex with the AR in the presence of hormone to upregulate its activity [44]. Bag-1L may act directly on the transcription factor complex as it was found to associate with androgen response elements in the absence of hormone. With recruitment of AR and Hsp70 to the PSA promoter, Bag-1L could perhaps assist these proteins in conformational changes that enhance interactions between the NH2-terminal and the COOH-terminal regions of the AR [24]. Bag-1L could also contribute to an increase in transcriptional activity through the recruitment of coactivators to the transcription regulatory complex [24], as well as potentially facilitate the cross-talk of AR with alternate signaling pathways via its association with

kinases [43]. However, this stimulatory effect of Bag-1L on AR activity cannot be generalized to all steroid hormone receptors. In contrast to AR, studies have shown that Bag-1L inhibits GR activity [45].

Cdc37, which was initially thought to be exclusively associated with kinase-chaperone complexes because of the lack of an association with GR [46], was later shown to directly interact with the LBD of the AR [40]. Cdc37 appears to influence AR transactivational activity in a partially hormone-dependent manner downstream of hormone binding [40,47], possibly by assisting the receptor in acquiring conformational changes required for activation. This effect cannot be inferred to all steroid hormone receptors as the loss of Cdc37 function did not have much of an effect on GR activation [47].

Additional chaperone proteins that have exhibited a role in AR transactivation activity, independent of their roles in previous folding processes, include Ydj1 as a stimulatory factor [48], and p23 as an inhibitory factor on AR activity. The inhibitory effect of p23 is yet another example of receptor-specific regulation since p23 was found to increase the transcriptional activity of GR [30]. Why these co-chaperone proteins exert differential effects with respect to the associated receptor is unknown. Even though steroid hormone receptors interact with the same minimal chaperone complex, it may be the absolute composition of the receptor-chaperone heterocomplex, with all of its associated proteins, that dictates the functional activity of the receptor.

5. Transcription complex disassembly (Fig. 1)

As mentioned above, the heterocomplex dissociates from the receptor once it has translocated into the nucleus. Yet, molecular chaperones remain implicated in receptor activity for the disactivation loop is believed to occur within the nucleus as well. A recent model proposed that molecular chaperones are involved in the disassembly of transcription factor complexes in order to promote the dissociation of hormone from the receptor and reassemble the receptor in the inactive state. Thus, if DHT is still present, reactivation of the AR occurs. However, if the hormone is no longer around, the receptor reenters the disactivation loop. In this manner, intranuclear cycling provides a transcriptional regulatory mechanism that is capable of detecting declining levels of ligand, resulting in the rapid termination of receptor activity once the signal is abolished [13,49]. In testing this disassembly model, it was revealed that p23, and possibly to a lesser extent Hsp90, actively disassembled regulatory complexes from DNA elements [50].

6. Degradation

Steroid hormone receptor degradation occurs via the ubiquitin-proteasome pathway [13]. When a receptor is unable to successfully transition into the high-affinity ligand-binding conformation the receptor undergoes degradation. CHIP (carboxyl terminus of Hsc70-interacting protein), an Hsp70 co-chaperone protein, has been shown to directly interact with the AR [51] and Hsp90 in the apo-receptor complex, causing the dissociation of p23 and loss of steroid binding ability [52]. Ubiquitylation is induced, followed by degradation via the proteasome [51–53]. CHIP-mediated degradation of the AR [51,53] has been put forth as a quality control mechanism to prevent protein aggregation [54]. CHIP may also prevent the accumulation of structurally unsound AR through the reduction in the rate of AR synthesis [53].

7. Chaperones in prostate cancer

An increase in certain chaperone proteins has been documented for prostate cancer. The basal cells of a benign prostate normally express Bag-1L, but the epithelial cells do not. However, in prostate cancer, the opposite is observed where Bag-1L is expressed in malignant epithelial cells and is no longer expressed in the basal cells. The distribution of Bag-1L is similar to Hsp70, which could possibly act synergistically to increase AR transcriptional activity [24]. Cdc37 is another chaperone protein that has displayed increased expression in carcinomas. Animal studies suggest a potential early role for Cdc37 in prostate cancer development. Overexpression of this protein in animal models has been associated with abnormalities such as prostatic epithelial cell hyperplasia and dysplasia [55]. In the human male, abnormal

expression of these proteins may act to enhance androgen receptor action providing a selective advantage to tumor cells during androgen ablation therapy [44].

8. Clinical implications

Alterations in the receptor-chaperone complex may well lead to aberrant AR signaling that could contribute to prostate carcinogenesis [24]. As described above, overexpression of certain molecular chaperones would enhance androgen receptor activity. Whereas a downregulation of inhibitory chaperones such as CHIP, which has a putative role in maintaining low levels of AR in the normal prostate [51], would contribute to progression via an accumulation of the receptor, resulting in an upregulation of AR action.

However, even in the absence of a causal role for chaperones in prostate cancer development, molecular chaperones serve as potential therapeutic targets in prostate cancer treatment. Currently, Hsp90 is a therapeutic target in prostate cancer clinical drugs trials [56]. Inhibition of Hsp90 with drugs such as geldanamycin prevents the stable association of p23 with the receptor-chaperone complex, therefore, trapping the AR in an intermediate state [57]. The persistent interaction between Hsp90 and steroid hormone receptors shifts the balance between folding and degradation toward degradation [13]. Targeting chaperone proteins in combination with AR antagonists or radiation may have a synergistic effect in impeding AR activity in prostate cancer [32,58].

9. Conclusion

Molecular chaperones play key roles in the folding, translocation, activation, and degradation of steroid hormone receptors depending on whether the receptor is in its inactive or active state [33,48]. There is a core minimal complex involved in the folding of steroid hormone receptors. When activated, the conformational state of the AR may recruit specific TPR-containing proteins depending on its trafficking and/or functional needs [10,30,34]. Competition for the AR between stimulatory factors and inhibitory molecular chaperones such as p23 [50] could influence the rate of disassembly of the transcription factor complex and, in turn, have an impact on the receptor's ability to rebind to the promoter and initiate another round of transcription.

Altered expression and/or function of molecular chaperones have the potential of augmenting AR signaling, which would then contribute to prostate carcinogenesis. One of the main molecular chaperones, Hsp90, is already being pursued as a potential therapeutic target. However, due to the ubiquitous nature of this chaperone, the potential for side effects as a result of targeting this protein is great. To minimize potential toxicities, efforts may need to be focused on discovering and targeting co-chaperone and chaperone-associated proteins, which have specific functional effects on the AR, but do not disrupt the signaling pathways of the other steroid hormone receptors.

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THE ANDROGEN RECEPTOR: UNLOCKING THE SECRETS OF ITS UNIQUE TRANSACTIVATION DOMAIN

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Whereas the androgen receptor (AR) protein shares similarities in the structure of its DNA- and hormone-binding domains with other members of the steroid nuclear receptor family, the molecule in its unliganded form has a seemingly unordered amino-terminal transactivation domain unique to the AR. A comprehensive understanding of the specific sub-structures and protein-protein interactions inherent to this domain in both its inactive and activated states remains unachieved. Therefore, the malleability of this peptide region in accommodating the diverse repertoire of transcription-modulating AR cofactors creates a great challenge for those intent on generating relevant three-dimensional molecular models. The AR transactivation domain achieves this flexibility through a series of conformational steps dependent on the presence of cofactors that induce allosteric changes, and thus has evolved several conserved peptide motifs representing key protein-protein interaction surfaces. Elucidation of these signaling regions, including their involvement in inducing AR transactivation domain structural changes, is of foremost interest in understanding how the AR achieves its pivotal role in regulating the androgen signaling axis particularly during the progression of prostate cancer.

I. THE ANDROGEN SIGNALING AXIS

Androgens play a key signaling role in a broad range of physiological systems, from influencing the development and maintenance of the immune and neural systems to exerting more obvious control over determining gender-specific phenotypes associated with the male reproductive system and secondary male traits (reviewed in Gelmann, 2002). It is with regard to the physiology of tissues and organs making up the latter that much attention has focused on understanding how androgens mediate cellular growth and differentiation, and how departures from this normally highly regulated signaling axis can lead to abnormal cellular growth, and ultimately cancers of organs such as the prostate. The main androgen in men is testosterone (T), with the testes

producing about 80–95% of the circulating T and the adrenal glands producing the remaining 5–20%. Carried in the blood bound to sex hormone-binding globulin, T diffuses into the cell and is converted by 5?-reductase activity into dihydrotestosterone (DHT), the form of the hormone possessing more potent biological activity. Downstream effects of DHT within the cell are then mediated by the androgen receptor (AR) protein, which is present in the cytoplasm but held in an inactive conformation through associations with molecular chaperones such as heat shock proteins. On binding of DHT, the AR undergoes conformational changes that result in dissociation from the chaperones and activation of the receptor. The activated AR translocates into the nucleus, where it coordinates the localized recruitment of transcriptional cofactors to promoters of AR-target genes, thus regulating expression. AR-regulated genes may be positively associated with cellular growth, such as in the case of prostate-specific antigen (PSA), or may be involved in proapoptotic pathways, such as in the case of p21. Cells that are androgen sensitive require the presence of functional AR to properly respond to the hormonal signal. Clinical outcomes resulting from attenuation or loss of AR activity as experienced in patients with complete androgen insensitivity syndrome or in patients undergoing hormone ablation therapy demonstrate the singular importance of the AR in mediating the physiological effects of androgen.

II. THE ROLE OF AR IN PROSTATE CANCER

Prostate cancer is the most common solid tumor found in men from Western countries and is the leading cause of cancer-related in death in those populations. Nearly 70% of these cancers involve adenocarcinomas of the peripheral zone, one of four distinct morphological regions of the organ (reviewed in Van de Voorde, 1996). In contrast, enlargement of the prostate resulting from benign prostatic hyperplasia exclusively involves tissues of the transition zone. The development and maintenance of the prostate is androgen dependent, and thus traditional treatment regimens for prostate cancer have involved removal of the testosterone signal. The efficacy of this strategy was first observed in the 1940s, when it was established that men with metastatic prostate cancer experienced tumor regression in response to castration (Huggins and Hodges, 1972). At present, radical prostatectomy or radiation therapy may successfully treat men whose early-detected cancers are clinically confined to the organ capsule. For cases of advanced disease in which tumors have spread to surrounding tissues, hormone ablation therapy involving antiandrogens such as flutamide or bicalutamide have immediate beneficial effects on tumor growth (Taplin and Ho, 2001). However, these ablation strategies are essentially palliative, as within a few years inevitably the tumors return in forms that are more aggressive and unresponsive to the particular hormone treatment (Debes and Tindall, 2004). This condition, known as hormone refractory disease, tragically carries with it a very poor prognosis. Gaining an understanding of

the mechanisms behind disease etiology and subsequent progression from androgen dependence to independence are key focal points of ongoing prostate cancer research. Epidemiologic studies have revealed associations between the size of a polymorphic CAG microsatellite repeat region in the AR gene and the risk of developing prostate cancer, with shorter triplet repeats being associated with increasing risk of disease (Giovannucci *et al.*, 1997; Ingles *et al.*, 1997). This will be discussed in greater depth later in the chapter. Extensive screening studies have cataloged more somatic mutations in the AR of advanced prostate cancer than in any other gene, and it is now estimated that such AR mutations can be identified in up to 40% of advanced-stage cases, with over half of these localized to the AR transactivation domain (Buchanan *et al.*, 2001). It is important to keep in mind, however, that the majority of these somatic AR mutations are believed to emerge only in advanced disease as a result of pressures placed on the tumor by earlier treatments such as ablation therapy. The belief is that exposure to antiandrogens that initially halt the growth of androgen-dependent cells eventually selects for the outgrowth of cell clones containing one or more of the following attributes (reviewed in Balk, 2002; Buchanan *et al.*, 2001; Feldman and Feldman, 2001): somatic AR mutations that allow the receptor to respond promiscuously to other nonnative ligands, become intrinsically active, or interact inappropriately with other signaling molecules; amplified expression levels of AR that create hypersensitivity to lowered levels of androgens; and heightened activation of AR through mechanisms of posttranslational modification of the receptor that normally are tightly controlled-an example would be AR phosphorylation via pathways stimulated by extracellular stimuli such as growth factors or cytokines (Debes and Tindall, 2004). Thus, although prostate cancer development and progression may involve several aberrant cellular pathways, it is abundantly clear that the activity of the AR represents a central hub through which these events pass. A discussion of how the androgen signaling pathways effect broad-ranging changes within the cellular context should begin first with an understanding of how the AR molecule is equipped to take the input signal of androgen and translate that into appropriate output signals that coordinate downstream transcriptional regulation. This chapter takes a closer look at the features of the AR that distinguish it from the other steroid nuclear receptors, with particular interest in discussing the functional roles of signaling motifs found within the transactivation domain of the molecule that are responsible for its activity.

III. OVERVIEW OF AR STRUCTURE

The AR is a 919-amino acid protein with a molecular weight of about 99 kDa encoded by a single-copy gene located on the X-chromosome at Xq11–12. The AR gene is approximately 80 kb in size and comprises 8 exons

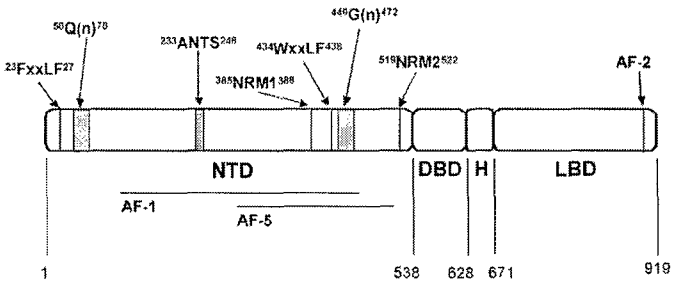

FIGURE 1. Functional map of the AR depicting the amino-terminal transactivation domain (NTD), DNA-binding domain (DBD), hinge region (H), and ligand-binding domain (LBD), with amino acid positions at boundaries. Location of the key signaling motifs discussed in the text are indicated.

that collectively possess a coding region of 2757 bp. Similar to other steroid receptors, AR consists of four main functional domains: part of exon 1 codes for the entire amino-terminal transactivation domain (NTD) amino acids ~1–538, exons 2–3 code for a DNA-binding domain (DBD) amino acids ~539–628, and exons 4–8 code for a hinge region amino acids ~629–670, containing a nuclear localization signal (NLS) and the ligand-binding domain (LBD) amino acids ~671–919 (see Fig. 1). For the sake of simplicity, the hinge region is often considered part of the LBD in models that choose to describe the AR as having three main functional regions (i.e., NTD, DBD, and LBD). Along these lines, it should be noted that there is no consensus in the literature as to which specific amino acid residues should be used to conceptually demarcate any of these domain boundaries; this is in part a consequence of the AR possessing polymorphic microsatellite repeats that have resulted in discrepancies in the early sequence publications. As a result, the reader should be aware that it is common in the literature for the AR to be described as consisting of 910 or 917 amino acids in addition to the 919 used in this chapter. Regardless, the numbering system used above is well suited to illustrate the modular nature of the protein and thus provides a convenient strategy for discussing the distinct functional roles carried out by these different domains.

IV. THE AR LBD

Substantial tertiary structural information has been obtained for the AR LBD in both its ligand-bound and ligand-unbound states, revealing that despite low homology in amino acid sequence, when compared with certain other steroid receptors, the three-dimensional structures adopted by the LBDs of these molecules show marked similarities (Sack *et al.*, 2001).

Steroid receptor LBDs generally consist of 12 alpha helices, and thus to follow convention, the AR helices are designated 1–12, even though the AR actually lacks the equivalent of what would be LBD helix 2 of the other steroid receptors. Ligands that bind the AR LBD do so by sitting in a ligand-binding pocket defined by helices 4, 5, and 10, which contact the A and C rings of the steroid molecules. On ligand binding, a conformational change takes place in the LBD, whereby helix 12, at the carboxy terminus of the AR, shifts to form a "lid" over the ligand-binding pocket, thus stabilizing the AR-ligand interaction. This has the secondary effect of revealing an otherwise inaccessible protein-protein interaction surface found at the AR carboxy terminal known as activation function 2, or AF-2 (Shiau *et al.*, 1998). This hydrophobic surface serves as the binding site for the p160 family of nuclear receptor coactivators, including TIF2, SRC1, and AIB1, which enhance AR-mediated transactivation through both direct modulations of AR conformation and recruitment of cofactors to the promoter-targeted AR that enhance transcriptional competence (Ma *et al.*, 1999). Another important role of the AR AF-2 motif is that of mediating interactions between the AR NTD and LBD; this AR N/C domain interaction is believed to be a functionally important event in receptor activation following ligand binding (He *et al.*, 1999). A precise understanding of how this AR N/C interaction mechanistically relates to receptor activation has yet to be defined, but ideas concerning its function are discussed in this chapter. For now, it is important to rationalize that the ligand-dependent nature of the AF-2 interaction surface thus imparts ligand dependence on a number of subsequent AR signaling events that occur through this LBD motif.

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V. THE AR DBD

As with the LBD, there is high functional conservation in the hinge region and DBD of the AR compared to other steroid receptors (Rundlett and Miesfeld, 1995). The main function of the hinge region is that of presenting an NLS that enables targeting of the activated AR to the nuclear membrane. This NLS signal in human AR spans amino acids 618–636 and has the sequence **RK**cyeagmtlga**RK**l**KK**lg. The functional consequence of disrupting this NLS has been demonstrated through experiments in which AR with mutations in this region has been shown to localize exclusively to the cytoplasm. Normal translocation of the receptor into the nucleus brings the AR in contact with target chromatin, where the receptor has high affinity for short conserved DNA sequences commonly referred to as hormone response elements found in promoter sites (Luisi *et al.*, 1991). These hormone response elements are specific for respective steroid receptors, and in the case of androgen response elements (AREs), consist of a pair of short hexameric half sites separated by 3-nucleotide spacer elements. The key

identifying feature of the two ARE consensus sequences thus far identified are conserved guanines and cytosines at positions 2 and 5 in each half site of the half-site pair, with only slight differences in the identities of other nucleotides making up the remainder of the sequences. The functional distinction between these two ARE types is not clear, but the relative positioning of these repeats, often in tandem, on AR-targeted DNA indicates that their combined role may not only be to specify the AR-binding site on DNA per se but also to coordinate receptor–receptor synergy of adjacently bound AR (Rastinejad *et al.*, 1995). In fact, the variation in number and relative orientation of AREs present in different AR-regulated gene promoters indicate that subtle modulation of how AR physically binds to the promoter DNA can influence how the receptors mediate transcription. The AR DBD recognizes these ARE sequences via two zinc finger motifs that are separately coded by exons 2 and 3 of the AR gene. These zinc fingers consist of about 25 amino acids each and configure a zinc ion bound to four cysteine residues at the base of each loop, in doing so positioning residues within the DBD for interactions with target DNA. At the base of the first loop there is a short peptide motif amino of acids 577–585, consisting of **G-S-c-K-V-f-f-k-R**, in this case with letters in bold indicating conserved residues among the steroid receptors. This interacts directly with cytosine and guanine at positions 2 and 5 of the target ARE half site. The second loop is involved in enhancing receptor-DNA binding through interacting with the first loop and may play a role in mediating receptor dimerization.

VI. THE AR NTD

The NTD of the AR makes up over half the amino acid sequence of the complete receptor, a distinction that sets the AR apart structurally from the other related steroid receptors that typically have relatively smaller transactivation domains. As discussed above, the separate functional domains of the AR act in a modular nature. Thus, when the reader regards the schematic representation of the AR domains (see Fig. 1), from right to left the interpretation is that the LBD acts as the on/off switch of the receptor, the hinge region contains the NLS signal responsible for transport of the AR into the nucleus, and the DBD serves as the anchor that positions the AR properly on the promoters of target genes. The role of the NTD, then, is that of subsequently conducting the coordinated recruitment of other proteins that will influence the transcriptional activity that takes place at the promoter, ultimately controlling gene expression. The NTD can be thought of as where the "action" of the receptor occurs, and in a sense the roles of the other AR domains are really to act as regulators of when and where the NTD is allowed to carry out its activity. The importance of the AR NTD is indicated not only by its size in relation to the other AR domains but also by its

complexity and lack of homology with domains of other steroid receptors. Because there is high structural homology of the DBD and LBD of the AR compared to other receptors, the substantial understanding of how these types of domains work in other steroid receptors can be applied to the AR. However, the structure of the AR NTD has remained elusive, primarily because of its being a largely globular domain that may require associations with AR-binding molecules to achieve its mature conformation (Reid *et al.*, 2002). The unique nature of the AR NTD invites much more scrutiny, and thus a detailed discussion of the features that comprise this domain follows.

VII. AR AF-1 AND AF-5

In general, The AR NTD transactivation activity is localized to two overlapping activation functions-AF-1 amino acids 142–485, and AF-5 amino acids 351–528 (Jenster *et al.*, 1995)-that encompass a number of peptide features such as microsatellite repeats, protein-protein interaction surfaces, and phosphorylation and sumoylation regulatory sites. The overall structures of these two generalized regions have not been determined, and it is believed that before receptor activation, the entire NTD may exist as a rather large fluid peptide domain in contrast to the highly structured DBD and LBD. On activation of the AR, associations with AR-binding proteins such as coactivators and transcription factors present at the gene promoter may collapse the NTD into structures that are optimal for the transactivation potential of the domain to be realized. Evidence of this mechanism has been shown in the case of the transcription factor TFIIF, which binds AR AF-1 in a manner that induces α-helical structure formation in the region (Kumar *et al.*, 2004). It is likely that this evolutionary approach of the AR NTD remaining unstructured before protein-protein associations allows for a variety of NTD structures to arise in response to different signaling contexts. One way to think about it is that if the AR is responsible for mediating the wide-ranging responses that cells exhibit to androgens, then clearly the manner in which the AR NTD communicates with other molecules must possess subtlety in its appropriate presentation of output signals. Thus, creation of interaction surfaces in the NTD, where the AR recruits downstream signaling proteins, is allosterically dependent on what upstream AR-binding partners are present to select for their formation. Interestingly, the actions of the two AF regions appear to operate with some independence, as the primary transactivation potential of the NTD in a full-length AR molecule is concentrated at the AF-1 region on ligand stimulation, whereas the focus is shifted to the AF-5 region in a truncated AR that lacks the LBD and is constitutively active. One implication of this finding is that the LBD has an inhibitory role on the NTD in the absence of ligand activation as a means of preventing inappropriate receptor activity, and

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there is evidence that this may involve recruitment of a peptide stretch found just upstream of the DBD in the AF-5 as a means of inhibiting AR-DNA binding (Liu *et al.*, 2003). This is one of several examples of specific peptide features within the AF-1 and AF-5 regions that control the NTD activity, and these will now be discussed in greater detail.

VIII. Q AND G REPEATS

There are two polymorphic trinucleotide repeats in exon 1 of the AR. The first consists of a stretch of about 21 CAG repeats coding for the amino acid glutamine (Q) spanning amino acids ~58–78, and the second is a stretch of GGN repeats that code for glycines (G) spanning amino acids ~449–472, with the majority of these codons being GGC. In the general population, the size distribution of the AR CAG allele ranges from 6 to 39 repeats but exhibits significant variation among the races, with 65% of African-American males possessing alleles shorter than 22 CAG repeats, compared to 53% of whites and 34% of Asian Americans (Edwards *et al.*, 1999). Our group (Irvine *et al.*, 1995) showed that the presence of shorter CAG and GGC repeats was observed in a high-risk group of African-American men, corroborating previous findings of a genetic component to prostate cancer predisposition. In the laboratory, shorter repeat size has been shown to result in increased activity of the receptor (Chamberlain *et al.*, 1994; Irvine *et al.*, 2000), and likewise complete deletion of the CAG repeat results in a receptor that is much more active than wild-type molecules (Callewaert *et al.*, 2003). It is thus believed that the presence of the Q and G repeats provides inhibitory control over the AR NTD. There is evidence that, just as with similar trinucleotide repeats found in other genes, the AR CAG microsatellites have expand though the course of evolution (Rubinsztein *et al.*, 1995). Overexpansion in CAG repeat size has detrimental effects on AR signaling. Abnormal repeat sizes of ~40 or more CAG are associated with diseases such as Huntington disease and spinal and bulbar muscular atrophy, which is commonly called Kennedy's disease. This finding has been attributed to the fact that AR molecules with more than 40 Q repeats show markedly decreased transactivational activity in *in vitro* experiments, even though ligand-binding affinities of these molecules are not affected (Chamberlain *et al.*, 1994). In addition, we have recently (Buchanan *et al.*, 2004) provided evidence that the Q repeat size influences the N/C interaction abilities of the AR molecule, with the range of 16–29 Q repeats optimal for AR N/C activity being reflected in 90% of the population having alleles in this range. Parallels exist in the proposed function of the G (encoded by GGC) repeats, with expansions beyond the 16–18 G that represent 90% of the population, resulting in decreased AR function (Ding *et al.*, 2004). Thus, there may have been an evolutionary bias in the expansion of the CAG and

GGC repeats up to an optimal length to favor N/C interaction as a means of AR regulation.

IX. AR NTD SIGNATURE SEQUENCE

Throughout evolution, the human AR has maintained over 70% conservation of its DBD and LBD with those from other species. However, this conservation is not present in the NTD, which shares typically less than 50% homology. However, it is notable that a single 14-amino acid NTD sequence spanning amino acids 233–246 is absolutely conserved in the AR across all species for which coding sequence is known. This sequence, AKELCKAVSVSMGL, is not present in any of the other steroid receptors, and thus most likely, it is the product of convergent evolution among species. Considering the suggested functional importance of this sequence by its unusually faithful conservation, and the fact that the AR NTD signature sequence (ANTS) sequence lies within the AF-1 region, an important role of ANTS in regulating AR transactivation activity seems highly likely. There is evidence that ANTS serves as an interaction site for heat shock proteins that act to stabilize the AR in its inactive state, and thus may represent a built-in inhibitory region that represses or deactivates receptor activity (He *et al.*, 2004). Further understanding of the functional nature of this motif and identification of additional AR cofactors that use it for receptor interaction are needed.

X. FxxLF AND WxxLF MOTIFS

As mentioned previously, the p160 coactivators are recruited to the AR via the DHT-induced AF-2 interaction surface of the AR LBD. The p160s possess a series of short LxxLL peptide motifs (where L is leucine and x is any other amino acid), commonly referred to as nuclear receptor boxes (NR-boxes) that directly contact the hydrophobic cleft of the AR AF-2. The Wilson group (He *et al.*, 2000) identified two LxxLL-like motifs present in the AR NTD that are highly conserved across the AR of many species, and they demonstrated that these motifs mediate interactions of the NTD with the LBD via the same AF-2 binding site used by the p160s. The first is a ^{23}FxxLF^{27} motif (in humans FQNLF) that has greater affinity with the AF-2 than that of the p160s. Binding of the FxxLF with the AF-2 results in the aforementioned N/C interaction that may displace (out-compete) p160 binding to the AF-2. However, the p160 molecule may still remain associated with AR through alternate binding sites in the AR NTD, localized roughly to the C-terminal third of the NTD amino acids ~351–538 (Irvine *et al.*, 2000; Ma *et al.*, 1999). The role of N/C interaction is not clear, but the

prevailing hypothesis is that this conformational change in response to DHT binding facilitates the activation of the receptor by revealing the protein-protein or protein-DNA interaction surfaces that subsequently result in dimerization, DNA binding, and receptor transactivation. Similarly, the ^{434}WxxLF^{438} motif (in humans WHTLF) is also involved in mediating N/C interaction, albeit much less efficiently than FxxLF. This WxxLF-mediated interaction does not involve the LBD AF-2 but, rather, some other region of the LBD that is yet to be determined. However, the primary role of the WxxLF may be to recruit the p160s to the AF-5 region of the NTD, as evidenced by decreased p160-AR binding to the NTD following mutation of the WxxLF motif.

XI. PHOSPHORYLATION OF THE AR NTD

The newly synthesized AR quickly undergoes posttranslational modification via phosphorylation, with the resulting modified form of the receptor adopting a higher molecular weight of about 110 kDa. Although it has been observed that the AR is phosphorylated at about a dozen serine residues throughout the receptor, the majority of these sites are located within the NTD and include constitutively phosphorylated positions such as serine 94, as well as those that undergo phosphorylation associated with receptor activation such as at serine positions 16, 81, 256, 308, and 424, as described by the Weber group (Gioeli *et al.*, 2002). There is quite a bit of evidence to indicate that phosphorylation is a normal means of regulating AR signaling both directly and indirectly. Of particular interest are pathways that can potentially activate the receptor without the binding of ligand that may be amplified in advanced hormone refractory tumors. These pathways involve extracellular stimuli such as growth factors, cytokines, and HER-2/neu acting through multitiered kinase signaling cascades that result in AR phosphorylation (Wen *et al.*, 2000). Thus, these different, yet often overlapping, pathways have been extensively investigated for their regulatory roles on AR activity.

Activators of protein kinase A have been shown to stimulate expression of the AR-mediated PSA gene independent of androgen in prostate cancer derived cell lines. The dose-dependent increase in PSA observed in these experiments was conditional on the presence of AR, as evidenced by inhibition of expression when the antiandrogen bicalutamide was introduced into the cells to interfere with AR function (Nazareth and Weigel, 1996). It would be inaccurate, though, to state that the functional consequences of phosphorylation on AR activity are necessarily stimulatory. As an example, the cytokine interleukin 6 has been shown to be both an AR activator as well as a repressor in vivo (Jia *et al.*, 2003, 2004). In addition, serine 515, which is near the carboxyl end of the AF-5, has been shown to be a phosphorylation

target of the MAPK signal cascade. Interestingly, experiments conducted using forskolin, a known stimulant of the MAPK pathway, have yielded conflicting results; LNCaP prostate cancer cells, when treated with forskolin, have been shown to exhibit enhanced AR activity, as indicated by increased expression of endogenous PSA (Jia *et al.*, 2003), whereas this same cell line has been used to demonstrate that forskolin induces dephosphorylation of the AR, resulting in impaired ligand binding affinity (Blok *et al.*, 1998). Seeming discrepancies in interpreting outcomes of AR phosphorylation in the laboratory are certainly in part a result of the lack of understanding of how the different AR serine positions available for phosphorylation are individually relevant. It is also quite likely that the overlapping nature of kinase cascades within the cell make it difficult for researchers interested in characterizing these pathways to isolate specific effects. Further work is needed to elucidate the complex nature of these mechanisms that act on the AR, but in the meantime it is clear that research focused on these nonsteroidal modes of AR activation may provide great insight into the progression of prostate cancer from androgen dependence to hormone refractory disease.

XII. SUMOYLATION AT NRM1 AND NRM2

The AR is posttranslationally modified by the addition of the small, ~100-amino acid protein SUMO-1 at two lysine residues in the receptor in a process referred to as sumoylation (Poukka *et al.*, 2000). These two NTD sites were originally identified as negative regulatory motifs, and thus are referred to here as NRM1 (^{385}IKLE^{388}) and NRM2 (^{519}VKSE^{522}), both of which exhibit sequences closely matching the consensus for targeting by the Ubc-type enzymes that serve as SUMO-E3 ligases (Iniguez-Lluhi and Pearce, 2000). This process is highly reminiscent of ubiquitination, in that homologous E1, E2, and E3 enzymes facilitate the activation and transfer of SUMO-1 to appropriate target substrates. Unlike ubiquitination, however, which results primarily in the irreversible flagging of proteins for degradation via the 26S proteosome machinery, sumoylation is a reversible process that mediates several different effects on targeted proteins. Such outcomes include activation, repression, intracellular localization, and degradation.

In the case of AR, proteins belonging to the family of protein inhibitors of activated STAT (PIAS) serve as SUMO-E3 ligases. Two members of this group, PIAS1 and PIASxalpha, have been shown to repress ligand-dependent transactivation of AR concordant with enhancement of receptor sumoylation (Nishida and Yasuda, 2002). Interestingly, the activation of the AR by ligand binding appears to be a prerequisite for sumoylation, a finding that implicates the covalent attachment of SUMO as a means of deactivating

AR molecules that are already engaged in transcriptional signaling. Deletion of the NRM1/2 sites in the NTD relieve the AR from SUMO-mediated repression, as does the activity of the AR-interacting protein Zimp10, which collocates with SUMO-bound AR and acts as an AR coactivator (Sharma *et al.*, 2003). Although the current understanding of AR sumoylation still leaves much to be desired, it is clear that this mechanism represents another layer of regulatory control targeted at the NTD; the positioning of these NRM motifs in the AF-5 subdomain raises additional questions as to the signaling properties of this receptor region.

XIII. AR NTD-ASSOCIATED COFACTORS

If it is true that the main function of the AR-NTD is to provide a staging platform for cofactor recruitment at the promoters and enhancers of target genes, the main questions are, What are these cofactors, and how do they function? A number of cofactor candidates can be considered (a subset of all reported ones) based on the fact that they are known to interact with the AR NTD and that they make physiological sense in terms of AR activity modulation.

A. p160 COACTIVATORS

Three related proteins with multiple names (SRC-1, GRIP1/TIF2, and pCIP/ACTR/RAC3/AIB1/TRAM1) belong to this family and collectively are the best characterized of the nuclear receptor coactivators (reviewed in Rosenfeld and Glass, 2001). Each one has multiple LxxLL motifs, which bind to the AF-2 region of all nuclear receptors. In the AR, an additional strong interaction surface for binding of p160 coactivators exists in the AF-5 region of the AR (Irvine *et al.*, 2000). It is therefore possible that p160 cofactors bridge AR N/C interactions.

B. BRCA1

Mutations in BRCA1 have been associated with familial breast cancer susceptibility. Although functions for BRCA1 in DNA repair have been proposed, it was shown that BRCA1 represses the activity of the ER (Fan *et al.*, 1999). However, we have demonstrated that BRCA1 functions as a coactivator for AR (Park *et al.*, 2000). In addition, we have shown that BRCA1 interacts with the AR primarily through the AF-5 region of the NTD (unpublished observations). Although the mechanism is unknown, this function of BRCA1 indicates that the AR may mediate the tumor-suppressor or growth-regulatory effects of BRCA1.

C. p300/CBP

These factors are secondary coactivators, normally recruited to the preinitiation transcription complex by primary coactivators such as the p160 family members. They provide necessary histone acetylation activity for the conversion of chromatin to transcriptionally permissive local structures. In addition, binding sites for CBP/p300 exist in AF-5 of the AR. It is unclear whether p300 and CBP play redundant or complementary roles with respect to each other.

D. ARAS

Chang and colleagues (Rahman *et al.*, 2004; Sampson *et al.*, 2001) have identified AR-associated proteins that interact with the AR NTD (ARA24, ARA160/TMF). ARA24 or RAN interacts with the Q repeat and also might be involved in AR trafficking in/out of the nucleus.

E. RHOA EFFECTOR PROTEIN KINASE C-RELATED KINASE

Recently, protein kinase C-related kinase was identified as a transcriptional activator of the AR via nonsteroidal superactivation and physical interaction with the AF-5 subdomain (Metzger *et al.*, 2003).

F. SMRT

This corepressor, originally discovered by the Evans laboratory, acts to inhibit nuclear receptor-mediated transactivation activity (reviewed in Rosenfeld and Glass, 2001). It interacts with both the AR LBD and AR NTD (Dotzlaw *et al.*, 2002).

G. CYCLIN D1

This protein is a required component of the CDK4 complex that plays a role in cell cycle control via phosphorylation of the retinoblastoma tumor suppressor gene (Harbour and Dean, 2001). Cyclin D1 binds directly to the AR NTD and inhibits AR transactivation activity (Petre *et al.*, 2002). It is not known exactly which regions or subdomains in the AR NTD are necessary for cyclin D1 binding or how structural alterations in the AR might affect the binding of cyclin D1 and its AR inhibitory activity.

H. SHP

The SHP—short heterodimer partner—protein is an orphan receptor that lacks a DNA binding domain and was recently shown to interact with the AR NTD, causing an inhibition of AR-mediated transactivation activity

(Gobinet *et al.*, 2001). Apparently the inhibition is caused by competition with AR coactivators like GRIP1/TIF2.

I. TBL1

TBL1, an F box/WD–40-containing factor forms a crucial part of the corepressor/coactivator exchange complex required for transactivation activity of many steroid receptors, including the AR (Perissi *et al.*, 2004). It may serve as an adaptor for the recruitment of the ubiquitin-conjugating/19S proteosome complex and thus may play a vital role in modeling factors on/off from transcription complexes.

J. CHIP

This COOH terminus of the Hsp70-interacting protein was identified by the Wilson laboratory (He *et al.*, 2004) as interacting with the ANTS sequence in the AR NTD (see earlier). CHIP functions as a negative regulator of AR transcriptional activity by promoting AR degradation.

Collectively, these observations indicate that optimal AR transactivation requires interaction of AR with an appropriate assembly of AR associated cofactors, and that if they are expressed in an inappropriate manner, AR-mediated gene expression can be driven by nonsteroidal mechanisms in the absence or at extremely low levels of androgens. The focus of future research is to define their binding sites in terms of the sequence motifs referred to earlier and how they effect AR interdomain interactions and activity.

XIV. THE FOCUS ON AR NTD

The AR NTD is unique among steroid receptors both in its expanded size and lack of determined form before activation. Although the latter fact has made gaining insight into the various NTD signaling features challenging on the basis structural homology comparisons, it also indicates that the ability of the AR to coordinate wide-ranging cellular responses to androgens may be a direct result of highly evolved structural flexibility. Unraveling the functional mechanisms of the NTD, from the nature of the N/C interaction to the roles of receptor phosphoryaltion and sumoylation, is a goal of great importance if we are to better understand how the AR is able to act as the central unifying molecule in the varied cellular pathways of androgenic response. Likewise, we now understand that the AR may still be playing the primary signal-mediating role in cases of hormone refractory prostate cancer resulting from amplification of secondary, nonligand dependent pathways. This is currently a focal point of research concerned with explaining how certain tumors that start as androgen-dependent tumors

can progress beyond the need for hormone stimulus with tragic results. The sheer number of new incidence cases of prostate cancer every year translates into a physical, financial, and certainly emotional burden for men and their loved ones that is perhaps unrivaled in male medicine. It is of paramount importance that researchers continue to learn about not only the etiology but also the nature of prostate cancer progression that so often turns from seemingly successful initial treatment to the rapid return of tumors that no longer respond to androgen ablation strategies. The AR is central to this research, and as we continue to gain better understanding of its molecular functions, undoubtedly we will see this knowledge translated into greater medical benefits.

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Endocrine Reviews

Interaction of Nuclear Receptors with Wnt/β-catenin/Tcf Signalling: Wnt you like to know?

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ABSTRACT: The cross regulation of Wnt/β-catenin/Tcf ligands, kinases and transcription factors with members of the nuclear receptor (NR) family has emerged as a clinically and developmentally important area of endocrine cell biology. Interactions between these signalling pathways result in a diverse array of cellular effects including altered cellular adhesion, tissue morphogenesis and oncogenesis. Analyses of NR interactions with canonical Wnt signalling reveal two broad themes: Wnt/β-catenin modulation of NRs (theme I) and ligand dependent NR inhibition of the Wnt/β-catenin/Tcf cascade (theme II). β-catenin, a promiscuous Wnt signalling member, has been studied intensively in relation to the androgen receptor (AR). β-catenin acts as a co-activator of AR transcription and is also involved in co-trafficking, increasing cell proliferation and prostate pathogenesis. T-Cell factor, a transcriptional mediator of β-catenin and AR, engage in a dynamic reciprocity of nuclear β-catenin, p300/CBP and TIF2/GRIP, thereby facilitating hormone dependent coactivation and transrepression. β-catenin responds in an equally dynamic manner with other NRs including the retinoic acid receptor (RAR), vitamin D receptor (VDR), glucocorticoid receptor (GR), progesterone receptor (PR), thyroid receptor (TR), estrogen receptor (ER) and peroxisome proliferator activated receptor (PPAR). The NR ligands, $VitD_3$, *trans/cis* RA, glucocorticoids and thiazolidines, induce dramatic changes in the physiology of cells harboring high Wnt/β-cat/Tcf activity. Wnt signalling regulates directly or indirectly developmental processes such as ductal branching and adipogenesis, two processes dependent upon NR function. β-catenin has been intensively studied in colorectal cancer, however, it is now evident that β-catenin may be important in cancers of the breast, prostate and thyroid. This review will focus on the cross regulation of AR and Wnt/β-catenin/Tcf but will also consider the dynamic manner in which RAR/RXR, GR, TR, VDR, ER and PPAR modulate canonical Wnt signalling. Whereas many commonalities exist by which NRs interact with the Wnt/β-catenin signalling pathway, striking cell line and tissue specific differences require deciphering and application to endocrine pathology.

Outline

I. Introduction

II. The Wnt/β-catenin/Tcf Signalling pathway

Activation and inactivation of the Wnt pathway (Figure 1)

III. Wnt/β-catenin/Tcf Modulation of Nuclear Receptor Function

a. Wnts
b. β-catenin
c. Cyclin D1
d. Glycogen Synthase Kinase β

Wnt/β-cat/Tcf modulation of NR Function (Table I)

GSK3β: a key regulator of Wnt and NR function (Figure 2)

IV. Nuclear Receptor Repression of Wnt/β-catenin/Tcf axis Signalling

a. Androgen, Retinoid Acid & Vitamin D Receptors
b. Glucocorticoid Receptor
c. Thyroid/Retionid X Receptor

Nuclear Receptor Modulation of β-cat/Tcf signalling (Table II)

Mechanisms of Nuclear Receptor Inhibition of Wnt/β-cat/Tcf function (Figure 3)

V. Summary and Therapeutic Implications

Abbreviations:
ADF2, adipophilin
AF-2, activation function-2
AI, androgen independence;
APC, adenomatous polyposis coli;
AR, androgen receptor;
ARE, androgen response element;
β-Cat/Tcf4, β–catenin/T-Cell Factor;
CBP, Creb binding protein;
C/EBP, CCAAT/enhancer-binding protein
CREB, cAMP-response element binding protein
CTD. C-terminal Domain
DBD, DNA binding domain;
DEX, dexamethasone;
DHT, dihydrotestosterone;
EGF, epidermal growth factor
ER, estrogen receptor;
E_2, estradiol
FABP2, fatty acid binding protein2
FRAT, frequently rearranged in advanced T-cell lymphomas;
GBP, GSK3 binding protein
GCs, glucocorticoids
GR, glucocorticoid receptor;
GRE, glucocorticoid response element;
GSK3β, glycogen synthase kinase 3 Beta;
HMG, high mobility group;
ILK, integrin linked kinase;
IGF, insulin-like growth factor;
LBD, ligand binding domain;
Lef, lymphoid enhancer factor;
NR, nuclear receptor;
NTD, amino terminal domain;
PI3K, phosphatidyl inositol 3-kinase;
PPAR, peroxisome proliferator activated receptor;
PrCa, prostate cancer;
PSA, prostate specific antigen;
PTEN, phosphatase and tensin homologue deleted on chromosome ten;
RA, retinoic acid;
RARE, retinoic acid receptor response element;
SRE, steroid response element;
T_3, tri-iodothyronine;
Tcf, T-cell factor
TR, thyroid receptor;
TZD, thiazolidinediones;
VDR, vitamin D receptor;
VDRE, vitamin D receptor response element;
WIF1, Wnt Inhibitory Factor

I. Introduction

Nuclear receptors (NRs) form a class of transcription factors that is regulated by small lipophillic ligands that includes steroids, thyroid hormone, retinoids (Vitamin A metabolites) and Vitamin D_3 (1, 2). At least 48 types of nuclear receptors have been identified and are divided into two general subfamilies (3, 4). Type 1 receptors are characterized by the formation of homodimers and include: the androgen receptor (AR), estrogen receptor (ER), mineralocorticoid receptor (MR) and progesterone receptor (PR). Type 2 receptors are characterized by the formation of RXR heterodimers and include: the thyroid receptor (TR), Vitamin D receptor (VDR), retinoic acid receptor (RAR), retinoid x receptor (RXR) and peroxisome proliferator activated receptor (PPAR). NRs mediate their effects, not only by ligand binding and gene activation, but also by post-translational events, such as phosphorylation, that may be brought about by interactions with a diversity of signalling transduction pathways including MAPK, phosphatidylinositol-3-kinase/Akt (PI3K/Akt) and Wnt (5, 6).

Novel ligands of NRs are continuously being developed and include synthetic steroid agonists and antagonists, ligands that alter fatty acid synthesis, analogues of Vitamin A + D, ligands with anti-diabetic qualities, and ligands with anti-cancer attributes (7, 8). Therapeutic intervention is well exemplified by the use of retinoic acid (9) and tamoxifen (10), both of which function as chemo-preventative agents. Thus, NRs and their cognate ligands serve as potent regulators of development, cell differentiation and normal physiology but may also have important implications for pathologies such as cancer (11). Given the fundamental role that NRs serve in maintaining a normal cellular *milieu,* it is not surprising that NRs and their cognate ligands can functionally interact with potent oncogenic systems, such as Wnt, to elicit changes in cellular adhesion and oncogenesis.

It is generally appreciated that a loss of cell adhesion is concomitant with metastatic cancer of endocrine tissues (12, 13). As such, a large number of reports has appeared during the previous 5-10 years

pertaining to immunohistochemical distribution of cadherins and catenins in endocrine-related cancers, which have been subject to several comprehensive reviews (14, 15). However, the usefulness of immunohistochemistry to evaluate changing expression of cell adhesion molecules as a predictor of clinical outcome is frequently confusing and unreliable, suggesting that alternative approaches for analysis of endocrine/adhesion interactions are necessary. In the previous 4-5 years, there has been a tremendous expansion in the number of reports pertaining to the functional interactions between NRs and the canonical, Wnt signalling pathway cascade. Increased laboratory technology, enhanced generation of recombinant Wnts and the development of pertinent transgenic animals have made it clear that these interactions are of greater functional importance than previously appreciated. Evaluation of NR interactions with canonical Wnt/β-cat signalling will likely aid in supplementing knowledge obtained by immunohistochemical analysis of pathology specimens and systemic endocrinology.

The Wnt signalling pathway is pivotal to gene expression (16), cell adhesion (17) and tissue development (18). Wnt ligands and β-catenin/T-cell factor (Tcf) signalling are also potent initiators of human oncogenesis (19) such that mutations in regulatory molecules, including the adenomatous polyposis coli (APC) tumor suppressor and β-catenin, have been shown to be key predictors of cancer progression (20). With the detection of β-catenin mutations in primary prostate cancers (PrCas) (21) followed by the exciting identification of a functional AR/β-catenin interaction (22), a flurry of studies have evaluated the nature and diversity of ligand sensitive interactions between AR and the β-catenin/Tcf signalling axis. Recently, exciting evidence for an oncogenic role for AR in prostate cancer was described by way of a conditional, transgenic mouse model harbouring a single AR mutation (E231G), ultimately leading to metastatic PrCa (23). Increased AR expression has been consistently associated with development of cells that become supersensitive to androgen facilitating the development of an 'androgen-independent' phenotype. Also, increased AR expression is also necessary and sufficient to convert PrCa to an ablation resistant state (24). Therefore, anything affecting AR signalling, such as β-catenin, may be expected to

have profound effects on PCa progression and that other NRs may respond in an equally dynamic manner to alter endocrine pathology. Thus, it is with interest that we assess how members of the NR superfamily can be modulated by components of the highly oncogenic Wnt signalling pathway.

Wnt ligands, β-catenin, Tcfs, cyclin D1 and glycogen synthase kinase beta (GSK3β) have all been shown to alter NR function by events including transcriptional activation, repression and phosphorylation. Conversely, NRs and their ligands confer dynamic effects upon Wnt function, as demonstrated by their abilities to promote dramatic alterations in E-cadherin, β-catenin gene targets and Wnt regulated physiology. Furthermore, GSK3β, has emerged as a more promiscuous kinase than previously appreciated with the capacity both to directly regulated NRs, by way of post-translational modification, and indirectly by directing IGF-1 dependent accumulation of β-catenin.

The emergence of studies demonstrating cross-regulation of Wnt/β-catenin/Tcf signalling with that of NRs provides an enticing platform on which to evaluate alterations in cell adhesion and transcription potentially occurring during endocrine cancer progression. Thus, in this review we summarize and interpret recent reports implicating functional interactions between NRs, including AR, RAR, RXR, GR, TR, VDR, ER and PPAR, and Wnt signalling with respect to alterations in gene expression, pathology, translational relevance and potential therapeutic opportunities.

II. The Wnt/β-catenin /Tcf Signalling pathway

Canonical Wnt signalling is well understood for its ability to regulate cell-cell adhesion and cell cycle control. Autocrine and paracrine secretion of Wnt extracellular ligands, in part, facilitate a high degree of control by binding the transmembrane, cysteine-rich family of Frizzled family of receptors (25, 26). Wnt binding of Frizzled, results in receptor kinase action, phosphorylation of the cytoplasmic mediator, Dishevelled, inhibition of the multifunctional serine/threonine kinase, GSK3β, ultimately

allowing for accumulation of β-catenin (27). Extracellular modulation of Wnt signalling may occur through competitive binding of Wnt ligands by secreted Frizzled Related Protein (sFRP) (28) or Wnt inhibitory factor (WIF) (29). Importantly, only selected Wnts are capable of initiating tumorigenesis as exemplified by Wnts 1a and 3 (30, 31). β-catenin, a promiscuous member and main effector of the Wnt pathway, is found in at least three cellular pools: (i) the adherens junctions in association with the transmembrane receptor, E-cadherin, and the cytoskeletal linker α-catenin; (ii) the cytoplasm and (iii) the nucleus in association with other transcription factors (17). These pools of β-catenin associate with partners such as E-cadherin or Tcf, mostly by way of the 12 Armadillo repeats, each containing 3 such helices (32). Soluble and non-soluble cytosolic forms of β-catenin are strictly regulated by the proteosome/ubiquitination system that consists of GSK3β, axin and APC (33-37).

Upon increased cellular levels and nuclear accumulation, β-catenin binds the amino terminus of the HMG binding protein, Tcf (T-Cell factor), and promotes its interaction with target DNA sequences (A/T A/T CAAAG) (38) thereby promoting displacement of the Tcf repressors, Groucho and CtBP (39). This, in turn, leads to a concomitant recruitment of co-activators such as CBP/p300 (40), Brgl and CARM1 (41) and an overall de-repression of Tcf transcription (42, 43). Activation of the Wnt/β-catenin/Tcf signalling pathway **(Figure 1a)**, either by disengagement of the APC/axin/GSK3 complexing or by Wnt activation promotes induction of downstream gene targets such as cyclin D1 (44), c-myc (45), PPARδ (46), Tcf-1 (38, 47), matrilysin (48) and CD44 (49). Induction of these genes has dramatic effects on cell and tissue development and oncogenesis (50-54). Negative Wnt signalling **(Figure 1b)** occurs by CK-1 phospho-priming of β-catenin, GSK3β phosphorylation of free β-catenin at Ser 33, 37, 45 and Thr 41 (55) and complexing with APC. This allows recognition by the F-box protein, βTrCP, and ultimately degradation by the ubiquitin/proteosome pathway (56). β-catenin levels are also regulated by Siah, a p53 inducible component that functions independently of GSK3β to recruit APC to target β-catenin for proteosome degradation (57, 58).

The Wnt/β-catenin/Tcf pathway is strict in regulation; however, aberrations to key regulators provoke increased promiscuity and interactions of Wnt components with other key effectors resulting in oncogenic transformation. This has been classically exemplified in colon cancer whereby mutations in APC promote decreased regulation in β-catenin and the resulting formation of intestinal polyps as observed in Gardners syndrome (59). Mutations in Axin2/conductin are also associated with formation of intestinal cancers due to accumulation of β-catenin (60). Additionally, alteration of the β-catenin degradation domain results in its stabilization, increased association with other effectors of cell proliferation and nuclear accumulation (61). Though Wnt gain-of-function is most frequently paralleled with mutational inactivation of its degradative system, stimuli including growth factors or activating ligands may confer increased union with other pathways. This is clearly the case between members of the nuclear receptor superfamily and associates of the Wnt/β-cat/Tcf axis.

IV. Wnt/β-catenin/Tcf modulation of NR function

a. Wnt

In addition to serving as powerful morphogens, Wnts function as regulators both of normal and pathological endocrine function. Several striking examples exist in which a strict requirement for changing Wnt expression is needed in order for morphogenesis to occur in tissues regulated by PR, ER and PPAR. For example, Wnt-4 expression is required, in a progesterone dependent manner, for mammary gland development as exemplified by the fact that Wnt-4 -/- mice fail to form ducts upon glandular transplantation to Wnt 4 +/+ mice (62). Wnts are also potentiators of endocrine morphogenesis as mammary epithelium stimulation with ectopic Wnt-1 can overcome the genetic loss of PR. Wnt-1 overexpression can promote breast cancer, as demonstrated in MMTV-Wnt-1 transgenic mice that, when crossed with mice null for ERα, produce offspring that develop hyperplasia and tumorogenesis by age 36-48 weeks (63, 64). Nevertheless, it is also apparent that Wnt-1 cannot substitute for the role that ERα

serves in ductal morphogenesis (63, 64), That Wnt-1 can promote tumorigenesis in the absence of estrogens or ERα further demonstrates that estrogen independent mammary oncogenesis may be compensated for by growth factor pathways such as Wnt. Nevertheless, these data also imply that therapeutic inhibition of Wnt driven β-catenin/ER interactions may merely serve to re-route Wnt proliferative signals through other effectors of proliferation.

Determination of stimuli of androgen independent cell proliferation is, perhaps, the *cause celebre* of PrCa research. Significantly, Wnt3a has recently been shown to sensitize AR to non-androgenic ligands and to promote AR co-activation independent of androgens. Wnt3a also promotes nuclear translocation of β-catenin and can enhance cell proliferation and tumorogenesis of PrCa cells in the absence of androgens (65, 66). Wnt11, a non-canonical Wnt, provides negative regulation of AR in androgen dependent PrCa cell lines but not in androgen independent cell lines implying that negative regulation by Wnts, when lost, may also promote carcinogenesis (67). NR/Wnt synergism can also be demonstrated by interactions between Wnt-1a and RAR (68, 69). Co-stimulation of mouse mammary cells transformed with Wnt-1a and treated with RA have revealed synergistic activation of the gene targets including tumor necrosis family ligand, ephrin B1, stra6, autotaxin and ISLR (69). Furthermore, RA given to transplanted mammary tumors, derived from Wnt1 transgenic animals or colon cancer xenografts (lacking functional APC), result in up regulation of stra6. These data imply that retinoids may be useful for increasing the efficacy of immunotherapies targeted at oncogenic targets of Wnt transformed cells. Collectively, these data indicate that Wnts may not only promote synergistic actions with AR but may promote oncogenic behavior by altering NR ligand specificity.

The partnership between Wnts and PPAR not only demonstrates a compelling example of how a Wnt-NR partnership can be essential for tissue morphogenesis, but also pre-empts another theme put forth in this review, that NRs repress the Wnt/β-catenin/Tcf axis. In the presence of cognate ligand, PPAR,

including isoforms α, γ and δ, regulate growth, differentiation, apoptosis (70) and insulin sensitivity (71). Activation of PPARγ is regulated by association with lipophilic ligands, including endogenous prostaglandins or synthetic thiazolidinediones. In the presence of adipogenic stimuli, PPARγ/RXR heterodimers function to promote differentiation of preadipocytes to adipocytes (72). Though Wnt signalling functions as a promoter of pre-adipocyte growth and proliferation, it also functions as a potent inhibitor of adipogenesis. For pre-adipocyte differentiation to occur, critical modulation of Wnt signalling must take place (73-75). Mechanistically, a reciprocal interplay of inhibitory signals occurs between PPARγ and Wnt/β-catenin signalling, such that in the absence of adipogenic stimuli, Wnts 1 and 10 promote growth and cell proliferation of pre-adipocytes by activating the Wnt targets, cyclin D1 and c-myc, while simultaneously inhibiting PPARγ. Cyclin D1 and c-myc facilitate these effects by binding PPARγ and the C/EBPα transcription factor respectively. (44, 76, 77). The presence of adipogenic stimuli, such as troglitazone, promotes induction of PPAR and C/EBPβ to facilitate a mandatory reduction in Wnts 1 and 10 (Fig 3d). Expression of C/EBPβ and C/EBPα also coincide with phosphorylation of the β-catenin regulatory domain and its proteosome degradation (73, 74, 78). Inhibition of adipogenesis relies upon CK1/GSK3β phosphorylation of β-catenin and not the Siah/p53 axis of degradation (GSK3β independent) (Liu et al., 2004), though some have conjectured that PPARγ may also promote degradation of β-catenin by a pathway that is strictly proteosome dependent and APC/GSK3β/p53 independent (79). PPARγ activation by TZD is also capable of directly influencing cyclin D1 in a CREB dependent, β-catenin independent manner resulting in hepatocyte arrest (80). PPARγ possess tumor suppressor functions that have been proposed to be elicited by activation of PTEN, a lipid phosphatase and negative regulator of Akt/PKB (81, 82). These data imply that PTEN-mediated antagonism of PI3K/Akt signalling, and reduced inhibition of GSK3β, may serve as a means to enhance adipogenic stimulation. This mechanism is congruent with other reports that have implicated PTEN as a negative regulator of β-catenin *via* the Akt/GSK3β axis (83, 84). The liver X receptor α (LXRα) may also participate in

regulation of adipogenesis. However, unlike PPARγ, the LXRα appears as a potential negative regulator of adipogenesis, possibly by collaboration with β-catenin (75). However, these observations remain preliminary with more investigation being required before a functional partnership between LXR and β-catenin in lipogenesis can be assigned. In addition to being highly expressed in adipose tissue, both PPARδ and PPARγ are thought to serve important roles in colon carcinogenesis, specifically as immediate targets of Wnt/β-catenin/Tcf activation. β-catenin and Tcf may serve both to bind and coactivate PPAR driven response elements, thereby activating PPAR target genes including FABP2 (fatty-acid binding protein-2), ADF2 (adipophilin) and Keratin 20 (85). Also, increased expression of PPARγ is evident both in human colonic tumors (86) and in the APC^{Min} mouse model (85, 87, 88) . Importantly, synthetic agonists of PPARγ have also been shown to induce increased colonic tumour mass in APC^{Min} mice, suggesting a link between a high fat diet and carcinogenesis of the colon (88). Thus, while the role of PPARγ as a contributor to colon carcinogenesis is considered somewhat contentious (89), convincing *in vitro* and *in vivo* evidence suggests PPAR isoforms are activated and over expressed in colon cancer systems.

b. β-catenin/Tcf

Evaluation of interactions, which are either direct or complexed, is an important prerequisite to understanding the functional significance of NR/Wnt crosstalk. Most nuclear receptors contain an N-terminal transactivation domain, a conserved DNA binding domain (DBD) and a carboxy region containing the ligand binding region (LBD) (90, 91). Whereas AR has an NTD AF-1 subdomain serving as the predominant site of transactivation, other NRs mainly use an AF-2 transactivation region found in the LBD (90). AF-1/2 regions are known to generate interactions with numerous transcriptional co-activators and repressors (92-94).

AR and β-catenin interact both by direct binding and complexing as ascertained by yeast 2-hybrid analysis (95, 96), GST pull downs (95, 97, 98), co-immunoprecipitations (22, 95, 98) and by transcriptional reporter assays (22, 95, 96, 98, 99). Other NRs that have been demonstrated to interact with β-catenin include the RAR (100) (101, 102) (103), VDR (104), RXR (105), PPAR (79, 80, 85, 106) and most recently ER (107). To date, however, neither the progesterone receptor (PR), glucocorticoid receptor (GR) nor thyroid receptor (TR) have been shown to directly interact with β-catenin (97, 99). Despite this, NRs failing to directly associate with β-catenin, in many instances, interact with other members of the Wnt signalling pathway including Wnts, GSK3β, cyclin D1, Tcf4 and Tcf1.

AR/β–catenin interactions are ligand sensitive, whereby complexing occurs in presence of dihydrotestosterone (DHT), or R1881, less in the absence of ligand (22, 98) and not in the presence of the pure AR antagonist, Bicalutamide (96, 98). Transient transfections of deletion mutant expression plasmids and yeast two hybrid studies suggest that the AR_{LBD} is necessary and sufficient for AR/β-catenin interactions (95, 96). Reduced AR/β-catenin binding, in the presence a pure AR antagonists, may be explained by formation of unfavourable stoichiometry by helix 12 of AR_{LBD} with respect to its binding pocket, thereby preventing efficient co-activator binding (108, 109). Despite the conservation in structure between NRs, it is clear that the AR_{LBD} has unique structural aspects that facilitate binding to β-catenin. LXXLL binding motifs (L= leu, X= any amino acid) contained within the AR_{LBD} serve a different function than most other NRs. In AR, LXXLL binding regions of the LBD serve to mediate NTD (containing FXXLF) and LBD interactions (110, 111), whereas in most other NRs, LXXLL binding motifs serve primarily to recruit transcriptional co-activators (112). Mutation of AR_{LBD} helices 3 and 12 result in disruption of AR/β-catenin binding, with alteration of helix 3 affecting binding of all three, β-catenin, the AR_{NTD} and the transcriptional regulator, TIF2 (Transcriptional initiation factor) (96). If β-catenin/AR_{LBD} binding depends on functional LXXLL binding motifs found within the AR, it is conceivable that β-catenin is required for changes in structural conformation coincident with ligand binding and dissociation

of Hsps. Regions of β-catenin necessary for interaction with AP have also been well defined. β-catenin Arm repeats 1-6 are required as mutation of repeats 5 or 6 abolish binding, co-activation and nuclear co-translocation interactions with AR (95). Importantly, Arm repeats 5 or 6 also bind Tcf4 and E-cadherin (32, 113) suggesting the therapeutic possibility of simultaneously disrupting the coactivating effects that β-catenin may confer upon either AR or Tcf4. That overexpression of Tcf4 or E-cadherin blocks β-catenin interactions with the AF2 region of AR suggests that β-catenin binds these molecules in close proximity of the Arm repeats (96, 98). β-catenin also contains LXXLL motifs, found on the second alpha helix of Arm repeats 1, 7, 10 and 12 (32, 114). However, deletion mutants of repeats 7, 10, and 12 suggest that these sites may not be necessary for AR/β-catenin binding (95, 96), as has also been suggested to be the case for interactions between the RAR and β-catenin (100). Structurally, this may be explained by the fact that leucine residues of the Arm repeats are buried within hydrophobic cores, possibly rendering them inaccessible to NR binding (114). ERα associates with β-catenin (107) and both Tcf4 and Tcf1 isoforms, though with varying physiological outcomes (115). While Tcf4 antagonizes ER, Tcf1 promotes transactivation of ER on estrogen response elements contained in an osteopontin promoter exemplify differential regulation between a NR and Tcfs isoforms and suggesting that the relative abundance of Tcf isoforms may dictate the physiology of consequence of Wnt stimulation (115). Genetic interactions between ER and β-catenin have been identified in Drosophila and can promote an estrogen-dependent, hyperplastic phenotype in the eye of Drosophila. Though these data imply that ER/β-catenin complexing may promote growth and tumorigenesis, the significance of ER/β-catenin interactions remain to be considered in other systems (107).

Correlated with the binding capacity of co-regulators is their ability to alter transcription. β-catenin is a potent transcriptional co-activator of AR (22, 41, 95, 96, 98, 116), RAR (100, 117), VDR (104) and ER (107). In accordance with the lack of binding observed between PRβ and GR (95-97, 100), β-catenin promotes little co-activation of these NRs (95). β-catenin, does however, transactivate AR both

on minimal transcriptional reporters (95-97, 118) as well as endogenous targets such as PSA (118) at a magnitude similar to CREB Binding Protein (CBP) (95) and SRC1 (22, 95), thereby demonstrating the potency of β-catenin as an AR regulator. Interestingly, β-catenin is more effective as an AR coactivator in cell lines harboring endogenous AR (119, 120) suggesting that the ability of β-catenin to enhance AR co-activation is sensitive to the presence of other co-regulators including CBP, a defined activator of β-catenin (40).

In general, the uniqueness of β-catenin/AR interactions are likely attributable to the unique structural identity of the AR_{LBD} but are likely also accounted for by differences in the supporting network of co-regulators between cell lines. Interestingly, while cells expressing high levels of β-catenin do not show increased VDR co-activation upon β-catenin overexpression, cells that express little nuclear β-catenin, elicit a potent $1\alpha,25(121)_2D_3$ dependent transactivation of VDR response elements (104). This effect could be attributable to the presence of high levels of nuclear β-catenin sequestering the available transcriptional machinery. In MCF7 breast cancer cells, activated forms of β-catenin (Ser37Ala mutation) have been shown to co-activate RAR, but not RXR, on RAREβ or MMTV-TRE luciferase reporter vectors. These data affirm that β-catenin activates only select NRs and that these interactions are likely dependent upon structural regions unique to each NR. The ability of β-catenin to serve as an AR activator likely also depends on variations in PI3K/Akt signalling status between cell lines. Constitutively activated PI3K signalling, found frequently in many PTEN -/- breast and PrCa cell lines, promotes decreased GSK3β function and, consequently, high levels of β-catenin (83, 84, 122). Therefore, AR co-activation may be less apparent in cells containing high levels of total β-catenin with relatively little opposition by the Wnt degradative system. System differences in β-catenin coactivation effects could also reflect the availability of AR pools to bind and import β-catenin to the nucleus (95, 97, 99).

Advanced PrCa is often treated by total androgen ablation therapy, however the ultimate phenotype is one of androgen independence and death (123). Transcriptional co-regulators are hypothesized to serve a critical role in promoting a more aggressive AR during AI PrCa. As such, several lines of evidence indicate that β-catenin may promote the oncogenicity of AR. Altered ligand responsiveness of AR has been postulated as a major mechanism by which PrCa continues to proliferate in low androgen environments (24). In light of this, it is significant that β-catenin increases AR-mediated gene activation not only in the presence of DHT, but also in the presence of the weaker adrenal androgen, androstenedione (22), a steroid remaining present in chemically castrated patients. Importantly, in addition to the AR specific co-activators ARA70 and ARA50, β-catenin is one of the few co-activators to enhance transcription in LNCaP cells upon treatment with 17β-estradiol (22). β-catenin also co-activates mutant forms of AR that are clinically relevant, including AR-W741C and AR-T877A, mutations found in PrCa cell lines isolated from hormone refractory PrCa patients treated with bicalutamide (AR W741C) (119, 124) and in lymph node metastatic lesions, respectively. These findings indicate that β-catenin acts as a co-activator both of WT and mutant AR and is not only altering the specificity of AR towards certain ligands but is also acting as a pure co-activator. The status of Wnt signalling in many PrCa cell lines (PC3, LNCaP) and bladder cancer (TSU) cell lines is low, likely due to relatively low levels of endogenous Tcf (98, 119, 120). Despite this, several lines of evidence indicate that β-catenin is important for progression of PrCa. Cre mediated excision of the β-catenin (exon3) regulatory domain develop hyperplasia and transdifferentiation in mice at 18 weeks of age but without metastatic behavior (125). In a similar model, stabilized β-catenin appears to be important for the initiation of prostatic neoplastic lesions (PIN) (126), a phenotype comparable to intestinal polyps, the precursor of invasive carcinoma and colon cancer. Also, fain-of-function, truncated forms of β-catenin occurring in metastatic prostate and breast specimens have been shown to preferential locate to the nucleus, possibly serving as an additional "pool" of β-catenin to promote cell proliferation during the androgen independent phenotype (127).

Genetic silencing of PTEN is a frequent genetic event in advanced PrCa and has been clearly associated with accumulation of nuclear β-catenin (83, 84) and cyclin D1 (76). Despite the loss of PTEN and accumulation of β-catenin, activation of β-catenin/Tcf gene targets is low in most advanced PrCa systems suggesting that the pro-proliferative effects of β-catenin are mediated *via* AR, as opposed to β-catenin's cognate receptor, Tcf .

AR/β-catenin interactions have distinct clinical relevance and therapeutic options. For example, β-catenin recruitment to AR is readily detected in a PrCa subline containing an AR-W741C mutation (isolated from hormone refractory PrCa specimens) (118), suggesting that β-catenin/AR complexing may be increased in hormone refractory PrCa. Furthermore AR/β-catenin interactions are reduced or abolished in the presence of AR antagonists suggesting that loss of β-catenin function may be associated with the beneficial effects of anti-androgen therapy for PrCa patients (118). β-catenin/AR_{LBD} interactions have been demonstrated to be dependent upon a single AR lysine (K720) for binding (96), a site also necessary for proper AR_{NTD} and TIF2 interactions (128). Small molecule targeting of K720 may serve as an attractive target, assuming that potential disruption of AR/TIF2 interactions can be tolerated. Certainly advances in peptidometics make this an appealing option, though the obvious challenge would be delivery without any severe systemic outcomes. Given that a moderate degree of homology exists between NRs that are known to bind β-catenin, including AR, RAR, RXR, VDR, ER, the obvious therapeutic challenge will be the tissue specific targeting of NR/β-catenin interactions. However, yeast 2-hybrid studies indicate that unlike AR, RAR does not bind the Armadillo repeats of β-catenin (101) emphasizing that differences exist in the manner by which NRs bind β-catenin. Further, whereas AR, VDR, and RAR have been shown to efficiently bind β-catenin, interactions between RXR and β-catenin are likely weak or transient as binding has been either difficult to detect (105), or not detected at all (100). Binding of NRs with β-catenin is likely not reliant upon NR_{DBD} regions, as several NRs, including GR and PR, have failed to demonstrate an interaction with β-catenin, in spite of the fact that most NRs contain near identical DBDs. These notions

suggest the possibility of targeting AR_{LBD} (helices 3,4,5)/β-catenin Arm repeat (1-6) complexes without detrimental effects on interactions that are mediated through other AR domains (95). Thus, small molecule inhibitory approaches targeting NR/β-catenin interactions are an enticing viable therapeutic approach for disruption of Wnt stimulatory effects on NRs.

c. Cyclin D1

Cyclin D1, a regulator of G0/G1 cell cycle progression, is a primary target of Wnt signalling and closely correlates with Tcf gene activation in many cell types (44, 50, 76). Cyclin D1 expression serves as a clinical predictor of poor prognosis in breast cancer (129, 130), ovarian cancer (131, 132) and thyroid cancer (133). However, most studies concur that upregulation of cyclin D1 in prostate adenocarcinomas is a rare cell cycle event and that cyclin D1 expression does not correlate with tumor grade or progression (134-136). Cyclin D1 is also poorly correlated with β-catenin signalling activity in PrCa cells (98). Despite this, an interesting relationship exists between AR and cyclin D1. Cyclin D1 can bind, ligand independently, to the AR_{NTD} resulting in repressed AR activity (137-139). It also promotes both cyclin dependent kinase (CDK) dependent mitogenesis and anti-mitogenic events dictated by the AF-1 domain of AR (138, 139). Mutational disruption of cyclin D1/CDK4 interactions do not, however, interfere with the repressive effects that cyclin D1 has on AR, suggesting cell cycle independent interactions (140). That AR expression is increased during advanced PrCa suggests that cyclin D1 may be progressively inhibited. Analysis of *in vivo* mouse models with stabilized expression of β-catenin recapitulate these observations and demonstrate that stabilized β-catenin induces little change in cyclin D1 levels but promotes significant increases in c-myc (126, 139).

As both AR and cyclin D1 are promoters of cell proliferation, further evidence will be required to decipher the significance of the apparent negative feedback loop that cyclin D1 confers upon AR. Regardless, cyclin D1 can be considered one of the few *bona fide* AR specific, co-repressors, as

demonstrated both in androgen dependent and independent environments (139). Interestingly, cyclin D1 also inhibits EGF and IGF, both of which are pro-survival factors leading to stimulation of androgen independent growth (139). As AR can repress β–Cat/Tcf signalling and its target, cyclin D1 (98, 120, 141), it is possible that endogenous cyclin D1 levels never achieve steady-state levels necessary to repress AR during progressive PrCa (Figure 2). It is tempting to speculate that cells with hyperactive Wnt signalling could incur negative selection against AR as suggested by the inverse correlation between the presence of AR and colon cancer (142). In contrast to AR and TR, cyclin D1 assumes a role as an activator of ER gene regulation (143-145) and prognostic factor for breast cancer (130, 146). Though ER/cyclin D1 binding requires an LXXLL motif in cyclin D1, this motif is not required for AR/cyclin D1 binding (144). By forming a trimeric complex with ERα and SRC-1 cyclin D1 can enhance estrogen transcription, estrogen dependently (143, 144). If cyclin D1/ER interactions are clinically important, than anti-estrogens disruption of these interactions may prove useful (143).

d. GSK3β

Glycogen Synthase Kinase-3β (GSK3β) resides at the junction of the PI3K/Akt (PI3K/Akt) and Wnt/β-catenin/Tcf survival pathways thereby serving critical roles in cellular metabolism, growth and proliferation (147, 148). Under non-stimulated conditions GSK3β pools are constitutively active but are phospho-inhibited upon PI3K/Akt or Wnt activation (27). While the substrates for GSK3β are generally specific and allow for disparate signals between PI3K and Wnt signalling (27, 147, 149) , it appears that GSK3β may not only directly regulate NRs but may also indirectly regulate AR function by modulation of PI3K/Akt and Wnt/β-catenin/Tcf pathways. Specifically, GSK3β both regulates cell cycle events in endocrine cancers but also post-translationally regulate NRs. For example, GSK3β binds and phosphorylates both AR (150, 151) and GR (152), at sites within the hinge and ligand binding regions resulting in decreased gene activation and cell proliferation. In accordance, overexpression of the GSK3β

inhibitor, Akt, or treatment with LiCl results in increased GR (152) and AR activity (150). This implies that endocrine pathologies, with mutational silencing of PTEN and Akt-inactivation of GSK3β may allow for more promiscuous NR function. siRNA mediated silencing of GSK3β results in suppression of androgen stimulated gene expression in PSA secreting cells (153). This suggests that while a minimal level of GSK3β is required for proper AR expression, higher levels, achieved in lesser-PI3/Akt sensitive cells, decrease AR activity. Interestingly, introduction of Akt resistant forms of GSK3β (deleted amino acids 1-9) has been reported to promote nuclear co-localization of GSK3β and AR (150), however, the physiological significance of this remains to be determined.

The dynamics of GSK3β/NR interactions cannot be properly interpreted without consideration of Akt, an upstream regulator of GSK3β and key dictator in the determination of how GSK3β influences AR function. Loss of PTEN expression and the resulting constitutive activation of PI3K/Akt signalling (154) (155, 156) results in repressed GSK3β function as frequently observed in PrCa and breast cancer (157-159). In these systems, GSK3β direct regulation of NRs is likely limited; however, in systems with reduced PI3K/Akt signalling, AR likely undergoes simultaneous inactivation by Akt/PKB and GSK3β–dependent phosphorylation with the net outcome, likely dependent upon the activity of PI3K signalling and the local concentration of androgens. GSK3β has also been demonstrated to mediate the co-activating effects that β-catenin confers upon AR, thereby reflecting the functional integration of Wnt and PI3K signalling in PrCa cells (83, 84, 160). Both Wnt3a (65, 66) and IGF-1 (insulin-like growth factor) (161) enhance AR co-activation and production of endogenous PSA. Therefore, an attractive hypothesis is that Wnt and PI3K/Akt growth factors phospho-inactivate GSK3β promoting stabilization, nuclear localization of β-catenin (98, 99), enhanced β-catenin/AR interactions (162) and enhanced proliferation (65, 66). Ultimately, however, it appears that the PTEN status dictates these events and that upon its loss creates an environment that is highly amenable for NR gene activation.

V. Nuclear Receptor Modulation of the Wnt/β-catenin/Tcf axis

Whereas Wnt/β-catenin stimulation promotes in general increase in gene activation of NRs, an equally conserved theme is the ability of NRs and their agonists to promote transrepression of the Wnt/β-catenin/Tcf pathway. This trend is observed not only by *in vitro* assays but also *in vivo*, thus suggesting NRs and endocrine pathologies harbouring activated Wnt/β-catenin signalling may be subject to therapeutic manipulation. Importantly, an increasing number of agonistic and antagonistic synthetic ligands are being identified that may influence the inhibitory actions that NRs appear to confer upon Wnt signalling. The modes are diverse and, in many cases, cell line dependent, however, it is clear that NRs promote the repression of Wnt/β-catenin/Tcf signalling.

a. Androgen, Retinoid Acid & Vitamin D Receptors

The co-activating effects that β-catenin exerts upon AR have been intensively studied, however, it is apparent that AR can invoke equally dynamic changes in Wnt/β-catenin function that include nuclear co-trafficking and transrepression. AR/β-catenin complexing and co-trafficking has been observed in PrCa cells containing both endogenous and exogenous AR (97). AR/β-catenin nuclear co-trafficking have also been reported in non-prostate cell lines such as CV-1 cells (95) and in pituitary cells (99). Furthermore, castrated mice reconstituted with androgen pellets show an obvious redistribution both of AR and β-catenin to the nucleus in normal prostatic epithelium (119). As β-catenin lacks a nuclear localization signal, AR provides a vehicle to recruit β-catenin to nuclear foci, sites likely consisting of active transcription (97). Consistent with binding requirements, β-catenin Arm repeats 1-6 have been shown to be necessary for efficient nuclear co-trafficking and resulting coactivation of AR in CV-1 cells (95, 99). However, functional $AR_{NTD\text{-}LBD}$ interactions or p160 co-activator binding are not required for AR mediated nuclear entry of β-catenin (99). Furthermore, GSK3β. or PI3K inhibitors fail to disrupt AR mediated nuclear accumulation of β-catenin (116). Together, these data suggest that there are minimal

structural requirements for AR mediated import of β-catenin and that this import is not dependent upon the β-catenin shuttling protein, APC (98) or the PI3K pathway (84). Recent studies have indicated that ligand-activated AR potently inhibits Wnt signalling in colon cancer cell lines and to a moderate extent in PrCa cells (96, 98, 99, 120, 141, 163). PrCa (CWR22-Rv1, LAPC-4, DU145) and non-prostate cell lines (TSU, HEK-293, SW480, HCT-116) that express AR demonstrate repression both of TOPFLASH and cyclin D1 protein levels upon treatment with DHT (119). In accordance with a mechanism of β-catenin reciprocity, increased Tcf expression and anti-androgen treatment can rescue the effects that androgens confer upon Wnt (98, 120). These studies strongly suggest that a limited pool of β-catenin can associate with either Tcf or AR in an androgen dependent manner.

AR repression of Wnt signalling is not without clinical relevance as pathological expansion of the AR polyglutamine tract (increases of 20 to 51 glutamine repeats) results in diminished, inhibitory effects on TOPFLASH reporter activity. These observations provide a potential connection between Wnt signalling and Kennedy's disease, a disease characterized by expanded glutamine repeats (141). They also implicate the expression of NRs with reduced colon carcinogenesis, a disease chacterized by heightened β-cat/Tcf activity. Though surmising a causal, *in vivo*, relationship between the presence of AR and reduced oncogenic β-cat/Tcf signalling is based on correlative data, it is interesting that non-cancerous, colon patient samples were scored positive for total AR expression at a higher frequency than those samples obtained from patients with colon cancer (142, 164). Interestingly, these studies did not observe any significant variations in colonic AR expression with respect to either sex and age of patients (164). Considering the reports documenting the repressive effects of NRs on β-catenin/Tcf signalling, it is tempting to possible that loss of AR, and possibly other NRs, contribute to aberrant Wnt signalling activity as observed in colon cancer cells. While speculative, the apparent ability of androgens to repress Wnt activity suggests the possibility that DHT analogues, targeting colon cancers, could reduce oncogenic Wnt/β-catenin/Tcf activity.

Retinoids are natural and synthetic derivatives of Vitamin A which regulate gene activation through the RAR/RXR nuclear receptor family (165). Importantly, retinoids have potent anti-cancer functions and have been shown to be effective in treatment of cancers of the lung (166), colon (167, 168) and prostate (169). Retinoid activated RAR is a potent repressor of β–cat/Tcf signalling in retinoid sensitive cells as exemplified in Caco-2 and HT29 colon cancer cells (100-102) and bronchial epithelium (170, 171). RA induction of E-cadherin expression, differentiation, and reduction of cyclin D1 may occur by diminishing Tcf sites of β-catenin (102, 103). However, in SKBR3 breast cancer cells the AP-1 pathway appears to be predominantly inhibited, rather than the Tcf axis (117), illustrating the broad manner by which retinoids exert their anti-cancer effects. Activation of the VDR with its metabolite ligand, $1\alpha,25(121)_2$ vitamin D_3, can repress Wnt/β-catenin/Tcf signalling and in some instances can promote dramatic alteration in the integrity of the adherens junction, increase differentiation (104, 117) and a resulting decrease oncogenic cell signalling (100). Using SW480 colon cancer cells as a template, reduced β-catenin expression and gene activation is achieved, by a means independent of APC and based on a mechanism of β-catenin reciprocity. This is supported by observations indicating that overexpression of WT Tcf-4, but not mutant β-catenin-non-binding-Tcf, can repress VDR response element mediated transcription (104). VDR mediated repression of Wnt target genes, PPARδ, Tcf-1, matrilysin, cyclin D1 and CD44, occurs prior to nuclear export of β-catenin, suggesting that a transcriptional event such as depletion of Tcf sites, pre-empts nuclear exit of β-catenin (104). Clearly, binding of β-catenin to VDR, Tcf and E-cadherin is competitive and $1\alpha,25(OH)_2D_3$ sensitive. $1\alpha,25(OH)_2$ vitamin D_3 induces increased VDR/β-catenin complexing in addition to enhancing E-cadherin expression and sequestering of Tcf bound β-catenin (104). Though VDR/β-catenin complexing and transrepression of β-catenin/Tcf gene activation is achieved in the presence of low concentrations (10^{-11}-10^{-7} M) of $1\alpha,25(OH)_2D_3$, repression of Tcf gene activation is VDR dependent as SW480 sublines (SW480-R and SW620 cells) not containing endogenous VDR show little $1\alpha,25(OH)_2D_3$ induced adhesion. The effects that RAR and VDR confer upon E-cadherin

transcription appear to be independent of regions regulated by the cadherin regulatory molecule, Snail (104, 172), suggesting that VDR ligands could induce E-cadherin gene activation, possibly by modulation promoter methylation status.

Given the potent transcriptional and morphological effects that RA and $1\alpha,25(OH)_2D_3$ can elicit in cells with hyperactive Wnt/β-cat/Tcf signalling, deciphering the mechanism by which E-cadherin is altered would appear to be of therapeutic importance. The combined use of demethylating agents with VDR ligands could be dually effective in enhancing differentiation and preventing invasive properties. $1\alpha,25(121)_2D_3$ analogues, including the deltanoids EB1089 and KH1060, demonstrate increased efficacy compared to $1\alpha,25(121)_2D_3$ in reducing β-catenin/Tcf reporter activity and, resultantly, are being used to treat neoplasia (173). Interestingly, clinical data supports an inverse correlation between $1\alpha,25(121)_2D_3$ vitamin D_3, dietary intake and sunlight exposure with the incidence of colon cancer (174, 175). Thus, it is tempting to speculate that this physiology could, perhaps, be explained by a dramatic redistribution of β-catenin from the nucleus to cell membrane, an effect exemplified in colon cancer cell lines in response to vitamin D_3 (104).

Reciprocal transrepression of the Wnt/β-catenin/Tcf axis by NRs is a mode of transcriptional repression that can be clearly applied to endocrine systems regulated by RAR, VDR and AR as supported by the following. RARα repression of β–cat/Tcf is concomitant with sequestering of shared co-factors with histone acetyl transferase (HAT) activity. Nuclear receptor deletion constructs, including AR ΔA/B (NTD deletion) or VDR ΔAF-2, dramatically reduce the ability of NRs to inhibit Wnt/β-cat/Tcf gene activation (101). Similar mutations in RAR (ΔA/B, ΔAF-2 and Δ408) do not inhibit TOPFLASH (101). That these altered regions are necessary for the co-activating effects that β–catenin confers upon NRs further implicates its role in a mechanism for NR transrepression. In addition to a reciprocal balance of β-

catenin, CBP/p300, a co-activator both for β-catenin (40) and many NRs (176, 177), has also been implicated as a major interacting protein between both Tcf and NRs.

b. Glucocorticoid Receptor

The glucocorticoid receptor and its ligand, dexamethasone, also modulate the integrity of E-cadherin/β-catenin complex, however, the mechanisms by which this occurs appears not to be by direct modulation of β-catenin/Tcf. Fascin, is a negative regulator of epithelial adherens junctions in mammary epithelial tumor cells (178). Upon exposure to dexamethasone, Fascin facilitates assembly of E-cadherin and β-catenin complexes (179, 180) resulting in enhancement of tight and adherens junctions (181-183). Thus, dexamethasone activation GR recruits β-catenin to the cell membrane to promote differentiation of mammary epithelium. Ligand activated GR also modulates the Wnt target, c-myc, through alteration of PI3K dependent pools of GSK3β. Inhibition of PI3K, using the inhibitors LY294002 and wortmannin, abrogates the ability of dexamethasone to modulate GSK3 (184), again affirming that PI3K dependent growth factors may cross regulate with NRs by convergence at GSK3β. GC and Wnt/β-cat/Tcf interactions have also been observed with changes in bone mineralization. In osteoblast cultures, GCs activate GSK3β, by way of a reduction in phosphorylated Akt^{Ser473}, thus promoting β-catenin degradation (185, 186). If Wnts contribute to osteoblast differentiation, then GCs appear to repress this morphological change, results that are recapitulated by the fact that mutations in the Wnt binding receptor LRP5, also appear to dampen Wnt promotion of osteoblast differentiation. That dexamethasone inhibits phosphorylated Akt, activates GSK3 activation and promotes β-catenin degradation further underscores that PI3K/Akt and Wnt pools of GSK3β can functionally interact (185, 186). GC modification of the adherens junctions also appears to be dependent upon changes in Ras (184), whereby alterations in Rho A may lead to an altered actin cytoskeleton including connecting partners, α- and β-catenin (187). With the ability to stabilize adherens junctions, GCs may be beneficial in restoring polarization, morphology or adhesiveness of poorly differentiated cancer cells.

c. Thyroid & Retinoid X Receptors

Wnt silencing is also observed in response to tri-iodothyronine (T_3) treatment in GC pituitary cells (188). T_3 can induce a striking time-dependent, inhibition of β-catenin that is concomitant with up regulation of axin levels (188). T_3 may also mitigates its inhibitory effects by downregulation of IGF, a known activator of the Wnt pathway (189). Activation of TRβ by T_3 promotes repressed β-catenin/Tcf and cyclin D1 gene activation in 293T cells and SW480 cells (133). Reduced levels of TRβ have been detected in colon cancers as compared to non-malignant samples, suggesting a selection against T_3/TR signalling in Wnt mediated oncogenesis. Undoubtedly, future studies will consider the effects that T_3 has on cell junctional morphology, growth and tumorigenesis. RXR also inhibits Wnt/β-cat signalling, however appears to follow a different paradigm than RAR or that of other NRs. The activating ligand, AGN194204, promotes RXR binding of β-catenin resulting in degradation and reduced β-cat/Tcf complexing (105), by a mechanism that is independent of APC but dependent upon steric hindrance of β-catenin (163). Thus, the mode by which RXR inhibits β-catenin appears to be distinct from the mode by which RAR, AR and VDR reduce activity of the β–Cat/Tcf axis. While RAR appears to decrease signalling by competition of nuclear co-factors, RXR operates by facilitating the degradation of β-catenin (105). The reasons for differential effects of RA treatment on Tcf transcription are unclear though cell line variation in basal β-Cat/Tcf activity, relative levels of cadherins and ratios of RAR/RXR or RXR/RXR dimers may be important. The effects of retinoids on Wnt signalling are not restricted to alteration of epithelial adhesion but can be extended to aspects of mammalian development (190, 191). Mammalian teratocarcinoma cells require increased Tcf gene activation during retinoic acid dependent morphogenesis and formation of the primitive endoderm (192). Retinoid mediated repression of several Wnt genes has

also been implicated as a required step in the differentiation of neuronal (NT2) cells (193). Thus, retinoids RA (all-*trans* RA or 9-*cis*-RA) appear to provide a two-fold benefit by reducing β-Cat/Tcf signalling and also up regulating E-cadherin levels. Stabilization of adherens junctions, in general, promotes sequestering of β-catenin to the plasma membrane and decreased Tcf gene activation (102, 117). These mechanisms, perhaps, contribute to the effectiveness of RA acting as a chemopreventive agent in cancers with hyperactive Wnt signalling (194, 195).

While nuclear receptors and their ligands are, in many instances, potent negative regulators of the Wnt/β-cat/Tcf axis, it is apparent that several mechanisms can be put forth by which NRs inhibit Wnt/β-cat/Tcf signalling including: (1) competition for a limited pool of β-catenin, and/or common co-factors, (between Tcf sites and NRs); (2) degradation of β-catenin and possibly recruitment of transcriptional repressors and (3) a direct interaction between NRs and Tcfs, promoting chromatin re-modelling. While cell line dependent differences in Tcf and NRs are likely functionally important, we favor the first scenario for VDR, AR and RAR for the following reasons: a) NR constructs with mutant β-catenin binding sites do not reduce β-catenin/Tcf signalling; b) NR-agonist mediated repression of β-catenin/Tcf signalling is relieved by overexpression of Tcf4; c) antagonized NRs does not impinge on β-catenin/Tcf signalling and d) changes in NR/β-catenin and Tcf/β-catenin complexing are observed as a function of ligand exposure. RXR, TR and PPAR appear to modulate Wnt/β-cat/Tcf signalling by a different mechanism, that is predominantly by way of GSK3 dependent phosphor-priming for proteosome dependent degradation (Figure 3)

Summary and Therapeutic Perspective:

The identification of functional interactions between Wnt signalling components and NRs is expanding rapidly. A growing number of NRs appear to be activated by β-catenin resulting in alterations in cell proliferation and tumorigenesis, whereas Wnt signalling appears to be compromised by the actions

of NRs. If these functional connections are truly important to endocrine diseases, then deciphering structural correlates of this reciprocal cross-talk between the pathways will be necessary for therapeutic assessment. Given the potential importance of β-catenin in PrCa and its ability to enhance AR function, the obvious therapeutic goal is to abrogate potential oncogenic AR/β-catenin interactions. Small molecules inhibitors targeting alpha helices 3, 4, and 5 of the AR_{LBD} or first six Arm repeats of β-catenin could prove effective. Alternatively, inhibition of extracellular growth factors, including Wnt3 and IGF-1, that are capable of β-catenin mediated activation of AR may serve as a viable option. Those endocrine pathologies harboring activated Tcf transcription could make use of viral delivery of vectors coding death genes (e.g. fadd) under the control of Tcf response elements (196). Mounting evidence suggests that mutational silencing of PTEN may promote β-catenin and AR gain-of-function. However, as PTEN loss appears to be a late genetic event in PrCa, small molecule inhibition of β-catenin may be most effective during late stage or androgen independent cancers, as opposed to early prevention. The ability of synthetic ligands to promote dramatic enhancement of junctional proteins and, thus, cellular differentiation suggests the potential for targeted use of NR ligands for treatment of Wnt activated cancers.

Recognized NR/Wnt interactions assume developmental roles and have implications for endocrine oncogenesis. A large body of evidence implicates β-catenin involvement in endocrine cancers; however, it also remains to be determined whether Wnt and β-catenin/Tcf complexing are initiators of oncogenesis or acting in concert with other signalling pathways in growth promotion. Important animal model studies, including the Wnt-1/ER knock-out mouse and prostate specific expression of stabilized β-catenin, do indicate that Wnt signalling can carry out functions necessary for endocrine oncogenesis. As activating β-catenin mutations provide gain-of-function, the development of pharmacological inhibitors could be applicable for inhibiting Wnt signalling in cells of endocrine tumors harboring such mutations. However, a therapeutic challenge is specificity as many inhibitor studies have targeted mutant forms of β-catenin but also non-specifically inhibit WT forms (197). Development of potent analogues, based on the properties

of AR, RAR, VDR, GR or PPAR ligands, may serve useful in endocrine cancers by reducing nuclear β-catenin while simultaneously increasing adhesion and differentiation. However, given that a variety of NR ligands can inhibit Wnt signalling it is tempting to speculate whether PrCa hormone withdrawal therapy could, in fact, enhance Wnt/ β-catenin signalling and cell proliferation, thus promoting androgen independent PrCa. This notion is consistent with data describing increased β-catenin levels in advanced PrCa tissue cores, most of which were obtained from patients who have undergone neoadjuvant hormone therapy (198).

The function of Wnt signalling is context specific as components of other major pro-survival pathways can clearly have major impact on normal and pathogenic Wnt signalling activities. While PTEN expression is lost only in some endrocrine cancers its capacity to alter NR transcription, promote cellular redistribution of β-catenin and induce expression of E-cadherin suggests that pharmacological mimicking of its tumor suppressor qualities may be of value in retarding NR transcription and anchorage independence. Abrogation of upstream PI3K/Akt signalling can be achieved by other regulators including SH2-containing Inositol Phosphatase (SHIP2) (199) and C-terminal modulator protein (CTMP) (200), regulators of phospholipids (PIP_3) and total Akt phosphorylation, respectively. Thus, it has become recently apparent that cross-regulation of GSK3β pools by PI3K/Akt or Wnt stimulation can promote altered AR function. Parallel convergence of PI3K/Akt and Wnt signalling upon GSK3β to promote β-catenin and AR gain-of-function is of potential interest. In these instances, activation of GSK3β may prove to have a beneficial effect reducing the growth promoting effects of cells harbouring activated Wnt and PI3K signalling. Cells that undergo simultaneous Wnt and PI3K activation would experience a tremendous selection against pools of GSK3β. That GSK3β is repressed in cells with loss of PTEN suggests that any increases in its expression could influence growth, cell cycle and oncogenic effects of PI3K and Wnt signalling. The therapeutic possibility is, therefore, for GSK3β activators or molecules capable of mimicking the tumor suppressor qualities of GSK3β pools, to degrade β-catenin and reduce NR

transcription. Clearly the therapeutic challenge will be to both (1) identify upstream regulators of GSK3β and (2) evaluate their potential for tissue specific activation.

The intersection of nuclear receptors and Wnt signalling is developing area but one of rapid expansion. Clearly, the means by which NRs interact with the Wnt/β-cat/Tcf axis are diverse and, in many cases, remain to be delineated. While the majority of reports are generated by *in vitro* systems, increasing implications to *in vivo* systems are being put forth. The ability of NR ligands to alter Wnt/β-cat/Tcf signalling in a manner which promotes dramatic changes in cell morphology and physiology provides motivation to provide answers for these outstanding questions. Determination of how NR signalling intersects with the Wnt/β-cat/Tcf axis to regulate cell cycle events that are both normal and pathological in nature will likely evolve into an of tremendous promise for development of targeted small molecule inhibitors and intervention.

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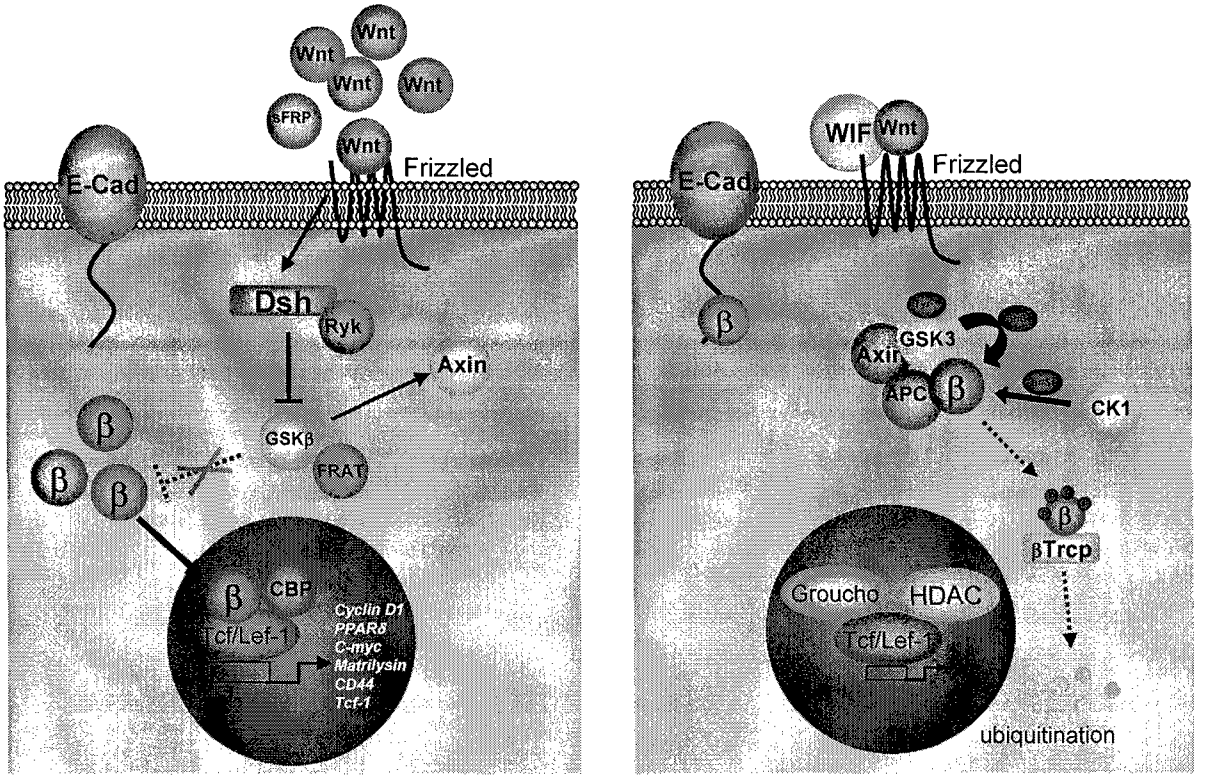
Figure 1: Activation and inactivation of the Wnt pathway. (a) Wnt ligands bind to Frizzled to activate Dishevelled (Dsh), phospho-inhibition of GSK3β, dissociation from Axin and binding to FRAT. Cytosolic β-catenin accumulates and translocates to the nucleus to activate Tcf/Lef-1 responsive genes resulting in gene activation and cell cycle progression. (b) Absence of Wnt stimulation results in the formation of an Axin/GSK3/APC complex, phosphorylation of β-catenin by casein kinase 1 (CK1) and GSK3β, followed by Trcp mediated proteosome degradation. Wnt target genes are, therefore, not activated.

Figure 2. Glycogen Synthase Kinase β: A mediator of AR regulation from Wnt and PI3K Signalling. Tumor suppressor qualities are contributed both by PI3K/Akt and Wnt pools of GSK3β. Endocrine cancers with elevated PI3K/Akt or Wnt/β-catenin signalling select against GSK3β activity, allowing for augmented levels of β-catenin available for ligand sensitive binding and transcriptional co-activation of AR. Cyclin D1, a downstream target of the β-catenin/Tcf signalling has been shown to bind and repress AR function, thus simultaneously generating promitotic and antimitotic stimuli. This results in reduced phosphorylation and increased co-activation by β-catenin. Mimicking the functions of PTEN or pools of GSK3β could serve to inhibit cell proliferation in PTEN -/- cancers

Figure 3. Wnt/β-cat/Tcf and Nuclear Receptors Engage in a reciprocal balance of ligand dependent activation and repression. **(a)** Interactions between β-catenin and NRs can be represented by two broad themes including β-catenin coactivation of NRs (theme 1) and NR repression of β-cat/Tcf (theme 2). **(b)** Cellular distribution of β-catenin is affected by ligand dependent activation of NRs and their cognate ligands including the VDR ($1\alpha,25\ (OH)_2D_3$); RARα (*trans*, *cis*-RA); AR (DHT); TR (T_3); GR (Dexamethasone) and PPARγ (TZD etc.). In the presence of ligand **(bi)**, β-catenin and p300/CBP associate in higher amounts with NRs, thereby promoting co-activation of SRE bound NRs. NR ligands can decrease nuclear levels of β-catenin and increase its association with E-cadherin. AR/β-catenin complexing and nuclear translocation is promoted in the presence of agonist. In the absence of ligand **(bii)**, more nuclear β-catenin is bound to Tcf, resulting in decreased transactivation on SREs. β-catenin and AR complexing is decreased in the absence of androgen. **(c)** TR represses β-catenin/Tcf activity by classical β-catenin degradative events, while RXR repression can occur by an alternative, APC-independent event. **(d)** PPARγ reciprocates with Wnt to dictate events that lead to either growth **(di)** or differentiation **(dii)** of adipocytes. Potent ligands such as TZD can relieve Wnt inhibition allowing for differentiation of adipocytes while also inhibiting growth *via* activation of PPARγ and its transcription factor partner, C/EBPβ.

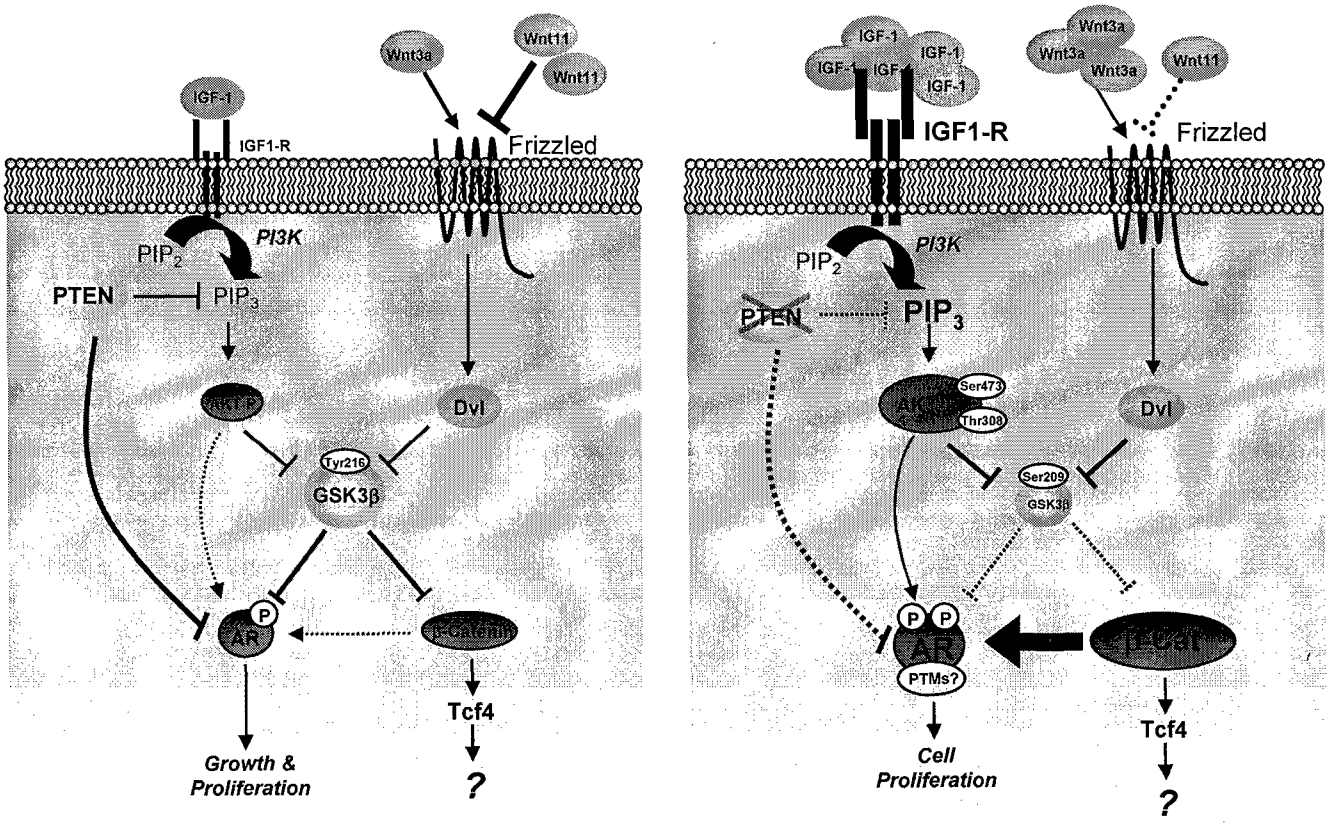
Figure 1



a) Activated Canonic Wnt Signalling

b) In-activated Canonic Wnt Signalling

Figure 2

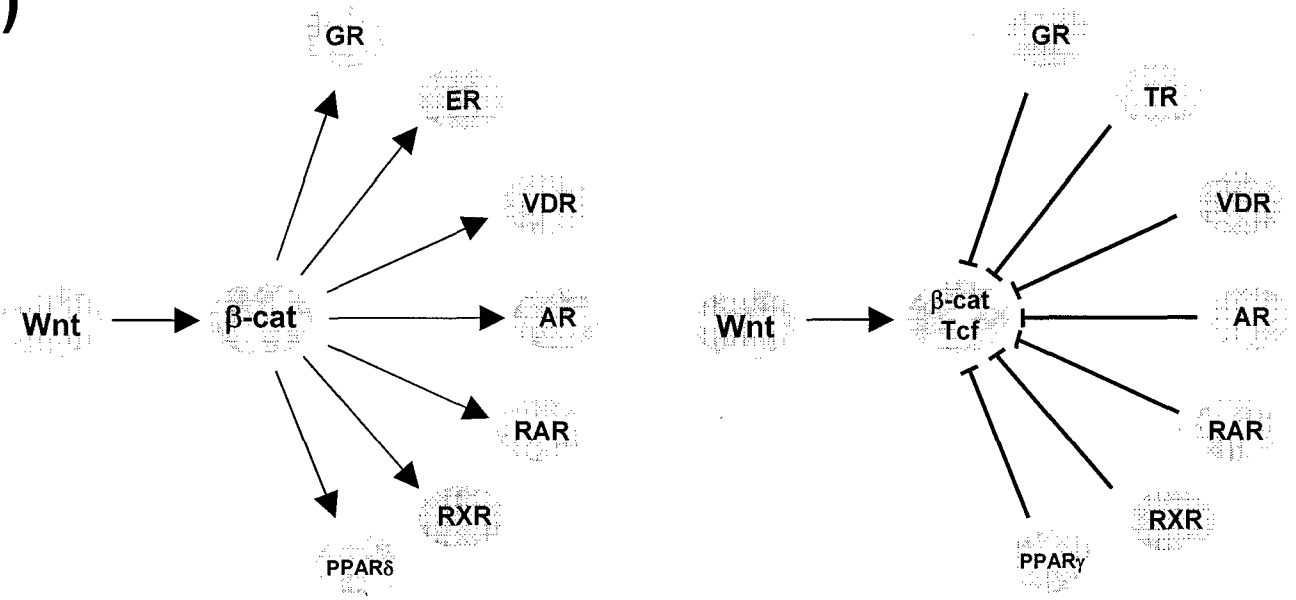


a) Androgen Dependent PrCa

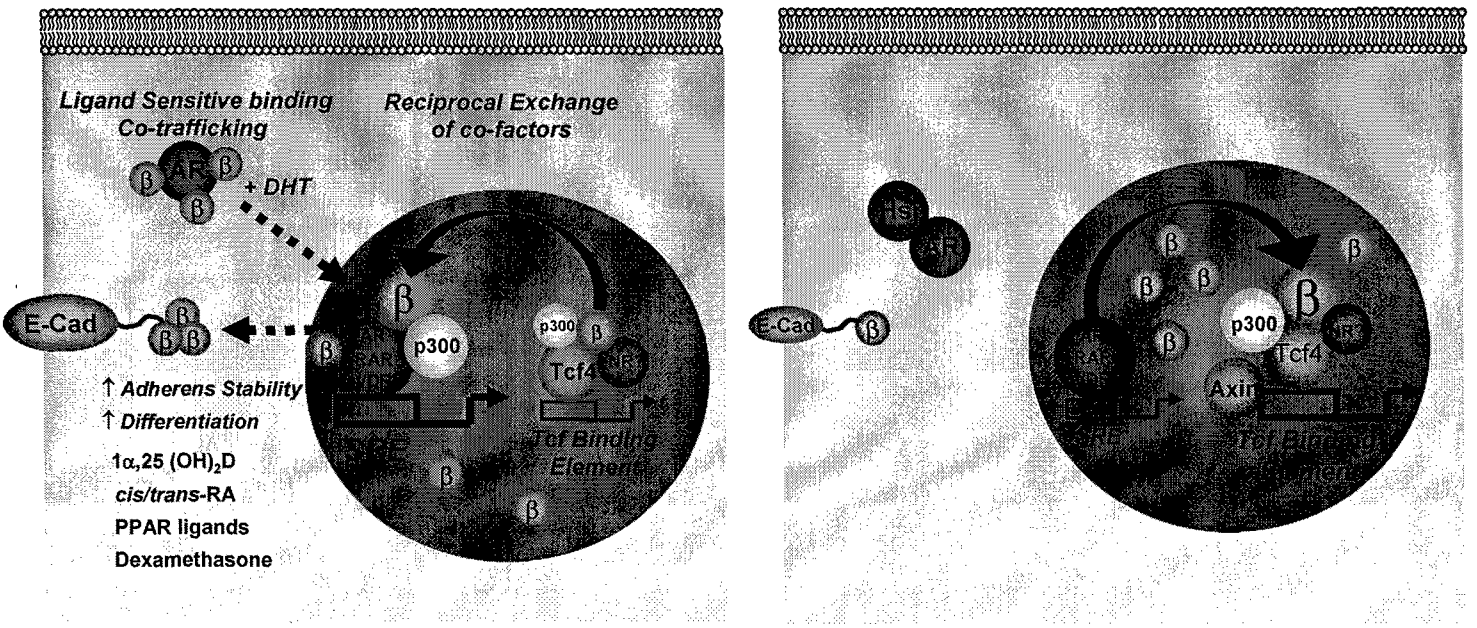
b) Androgen Independent PrCa

Figure 3

a)



b)

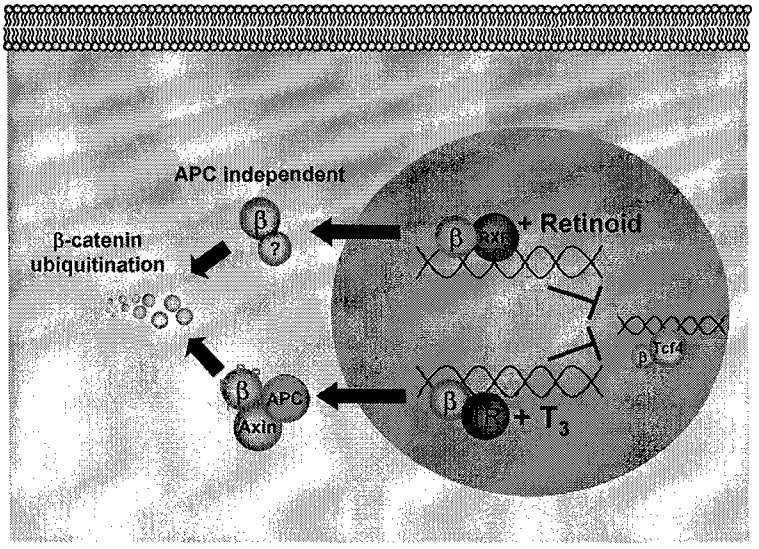


NR Transrepression of Wnt/β-Cat/Tcf Signalling

Basal Wnt/β-Cat/Tcf Signalling

Figure 3

c)



TR & RXR repression of Wnt/Tcf/β-cat

d)

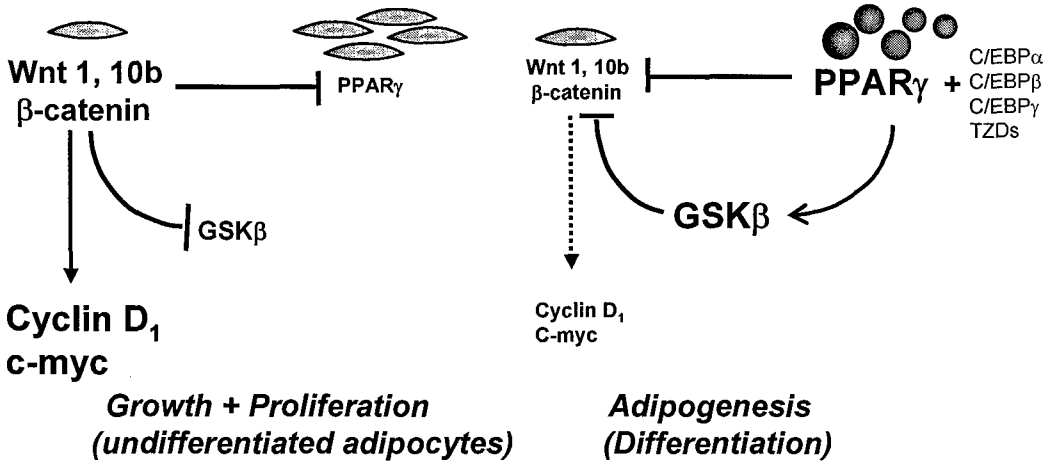


Table I: Wnt/β-cat/Tcf modulation of Nuclear Receptor Function

Wnt	NR	Function	Observation	Ref
Wnt-3a	AR	Activation	Promotes ligand, independent AR activation (LNCaP cells) ↑ Nuclear complexing of AR/β-catenin (LNCaP cells) ↑ cell proliferation, colony formation (LNCaP cells)	(65)
Wnt3a	AR	Activation	↑ cell proliferation in 22Rv1 and LNCaP cells	(66)
Wnt-11	AR	Activation/Inhibition	↓ AR activity in AD PrCa cells (LNCaP) No inhibition in AI PrCa cells (LNCaP-r) ↑ Wnt11 expression in AI cells (LNCaP-r) and xenografts	(67)
Wnt-4	PR	growth factor	essential function for Wnt4 in mammary morphogenesis	(62)
Wnt-5a	PPARγ	↓ gene expression	Inhibits preadipocyte differentiation in 3T3-L1 cells	(74)
Wnt-1	PPARγ	↓ gene expression		(78)
Wnt-10	PPARγ	↓ gene expression	Inhibits PPARγ and CCAAT/EBPα	(73)
Wnt-1	RA	↑ RARγ gene	Synergistic gene activation (e.g. Stra6) in transformed cells	(68)
β-catenin	AR	Co-activator, binding	DHT sensitive, MMTV-luc, Prob-luc PrCa (LNCaP, PC3) & bladder Ca cells (TSU) TSU cell in the presence of E_2 presence of androstenedione, DHEA	(22)
β-catenin	AR	Co-activator, binding	2xARE-luc, PSA-luc AR LBD sufficient for binding, β-catenin Arm repeats 1-6 CV-1, LNCaP cells E-cadherin modulates β-cat co-activation of AR	(95)
β-catenin	AR	Co-activator, binding	3xARE-luc LNCaP, PC3 cells	(97)
β-catenin	AR	Co-activator, binding	AR positive cells, PSA-luc, HK-2-luc Gene activation of endogenous targets NOT AR negative cells Ligand independent co-activation (TSU cells) Mut β-cat does not increase LNCaP proliferation	(119)
β-catenin	AR	Co-activator	DHT, hydroxyflutamide causes β-Cat recruitment L-39 and cyproterone acetate does not recruit β-Cat in LNCaPs Bicalutamide recruits β-Cat and activates AR in LNCaP-W741C Δβ-Cat/NTD GSK3β sites regulates *in vivo* PSA levels	(118)
β-catenin	AR	Direct binding, Complexing	β-Cat Arm repeats 1-6 necessary AR-LBD region necessary Cooperates with AR-NTD and TIF2 AR helices 3, 4, 5, + 12 required for b-cat binding β-Cat LXXLL motifs not required for AR/β-cat binding	(95) (96)
β-catenin	AR	Activation	Mutant β-cat activated AR in 22Rv-1, LNCaP cells NOT	(66)
β-catenin	VDR	Direct binding Complexing Co-activation	*In vitro* $1\alpha25(OH)_2D_3$ sensitive co-activation VDR metastatic colon carcinoma cells	(104)
β-catenin	RARα	Co-activator, binding	*In vitro* and *in vivo* (MCF-7, Hs578t, SKBR3, Caco-2, HT-29 cells) 9*cis*- & *trans*- RA dependent	(100) (101)
β-catenin	RXRα	No binding detected		(100)
β-catenin	RXRα	weak binding	cross-linking used to detect binding β-catenin by APC-independent mechanism	(105)
β-catenin	PPARγ	↓ gene expression	Inhibits adipogenesis	(106)
β-catenin	PPARγ	Activation	Promotes ↑ expression in APC^{min}	(85)
GSK3β	GR	Co-repression	Phosphorylation (P-Thr^{171}) dependent (rat NOT human)	(152)
GSK3β	AR	Co-repression,	Binding/phosphorylation to AR hinge region	(150)
GSK3β	AR	Co-repression	Phosphorylates AR-NTD, interrupts AR-NTD/CTD interactions	(151)
GSK3β	AR	Regulator	Required for androgen stimulated gene expression	(153)
GSK3β	PPARγ	Inhibition	Inhibits adipogenesis and mimics Wnt signalling in	(73)
Tcf-4	ERα	Co-repressor	*In vitro*, *in vivo* binding	(115)
Tcf-1	ERα	Co-activation	*In vitro*, *in vivo* binding, synergistic gene activation	(115)
Tcf-4	AR	↓ transcription	weak binding *in vivo* + *In vitro* to AR-DBD region	(163)
Tcf-4	AR	↓ transcription	↓ ARR3-Luc, weak binding in vivo	(97)
Tcf-4	PPARγ	Activation	Increased PPARγ detected in colonic cancer cells	(85)

Table II. Nuclear Receptor Modulation of Wnt/β-catenin/Tcf Signalling.

NR	Ligand	Effect on Wnt Pathway	Reference
AR	DHT, R1881	↓ β-Cat/Tcf gene activation	(96) (98) (99) (101)
		inhibits cyclin D1 (colon NOT PrCa cells)	(98)
		Inhibits colon cancer cell proliferation	(98)
AR	R1881	no change in β-cat levels in prostate cells	(119)
AR	R1881	*In vitro*, co-trafficking of AR & β-catenin (neuronal cell line)	(99)
		In vitro, co-trafficking of AR & β-catenin (LNCaP, PC3 PrCa cells)	(98)
		In vitro, co-trafficking of AR & β-catenin (CV-1 cells), necessary Arm repeat 1-6 are necessary for AR/β-catenin binding and trafficking	(95)
RAR	*all*-trans RA, 9cis-RA	↓ β-cat/Tcf gene activation (SW480, Caco-2, HT29, SKBR3, MCF7)	(100)
		↓ cell proliferation (SW480, Caco-2, HT29, SKBR3, MCF7)	(100)
		↑ activation of retinoid responsive genes and RARE reporters	(100) (117)
		β-cat/Tcf activation NOT affected (HCT116, SW480 cells)	(100) (117)
		CBP/p300 mediates transrepression of β-cat/Tcf	(101)
		↓ cyclin D1 independent of β-cat/Tcf (SKBR3, MCF7 cells)	(117)
		↑ E-cadherin expression	(100)
		↑ β-cat stability (MCF7, SKBR cells)	(100) (117) (102) (103)
RXR	*all*-trans RA, 9cis-RA	Ligand (AGN194204) dependent b-cat binding ↑ β-cat degradation, Hek293, HeLa ↓ β-cat /Tcf gene activation (CV1, SW480 cells) APC-independent inhibition	(105)
TH	T_3	↓ Wnt gene repression (Tcf1, CD44, PPARδ, ZO-1)	(188)
TH	T_3	↓ β-cat/Tcf & ↓ cyclin D1 transcription, ↑ β-cat degradation	(133)
ERα,	E_2	none reported	(115)
ER	E_2	transient GSK3 activation in Hippocampus	(201)
ER		Complexing of GSK3β, ERα and β-catenin in absence of E_2	
ER	E_2	WISP (Wnt Inducible Signalling Protein) is highly E_2 responsive	(202)
VDR	1α, 25 $(OH)_2D_3$	↓ β-cat/Tcf gene activation	(100)
		↑ E-cadherin and differentiation	(104) (117)
PPARγ	C/EBPβ + ligand	↓ β-catenin protein, ↓ β-cat/Tcf gene activation	(106)
PPARγ	Troglitazone	GSK3β dependent degradation of β-catenin	(203)
PPARγ		↓ cyclin D1 activity by depletion of p300/CBP	
PPARγ		↓ cyclin D1 activity independent of β-cat/CREB dependent	(80)
PPARγ2		β-catenin degradation independent of APC/GSK3β/p53	(79)
PPARγ		↑ E-Cad expression and membranous β-cat	(204, 205)
PPARγ		↓ Wnt-1, Wnt-10b inhibition of adipogenesis	(73-75)
GR	Dex	↓P-Akt-Ser 473, ↓P-GSKβ-Ser9, ↑ GSK3β activity	(185)
GR	Dex	↓ fascin, ↑ β-catenin, ↑ E-cadherin	(179, 184)
GR	Dex	↓ α-catenin, ↓ β-catenin, ↓ cell aggregation (235-1 pituitary cells)	(206)
GR	Dex	↓ c-myc activity by ↑ Thr^{58} phosphorylation (osteoblasts) ↓ Ser^{9} = ↑GSK3β kinase activity	(185)
ER	Wnt-1	ERα not required for Wnt-1 mediated tumourgenesis in ER (-/-)/ Wnt-1 transgenic mice	(63)