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Amino-Terminus Domain of the Androgen Receptor as a Molecular Target to Prevent the Hormonal Progression of Prostate Cancer

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Abstract Prostate cancer has a propensity to metastasize to the bone. Currently the only effective systemic treatment for these patients is androgen ablation therapy. However, the tumor will invariably progress to an androgen-independent stage and the patient will succumb to his disease within approximately 2 years. The earliest indication of hormonal progression is the rising titer of serum prostate specific antigen. Current evidence implicates the androgen receptor (AR) as a key factor in maintaining the growth of prostate cancer cells in an androgen-depleted state. Under normal conditions, binding of ligand activates the receptor, allowing it to effectively bind to its respective DNA element. However, AR is also transformed in the absence of androgen (ligand-independent activation) in prostate cells via multiple protein kinase pathways and the interleukin-6 (IL-6) pathway that converge upon the N-terminal domain of the AR. This domain is the main region for phosphorylation and is also critical for normal coregulator recruitment. Here we discuss evidence supporting the role of the AR, IL-6 and other protein kinase pathways in the hormonal progression of prostate cancer to androgen independence and the mechanisms involved in activation of the AR by these pathways. Receptor-targeted therapy, especially potential drugs targeting the N-terminal domain, may effectively prevent or delay the hormonal progression of AR-dependent prostate cancer. J. Cell. Biochem. 98: 36–53, 2006.

Key words: prostate cancer; androgen receptor; IL-6; androgen independence; metastasis; phosphorylation; protein-protein interactions

Prostate cancer constitutes a major health problem in western countries. It is the most frequently diagnosed cancer among men and the second leading cause of male cancer death. Furthermore, with an aging population the incidence of prostate cancer has quickly increased such that 50% of men over the age of 80 exhibit underlying prostate cancer [Jemal et al., 2005]. The initial treatments for prostate cancer are typically radical prostatectomy or radiation to remove or destroy the cancerous cells while they are still confined within the prostate capsule. However, approximately 20% of men treated with radical prostatectomy will experience tumor recurrence and 11% of men will already have bone metastases at the time of clinical presentation [Landis et al., 1998]. Most men succumbing to prostate cancer have osseous metastases. An examination of osseous metastases in 20 bone sites revealed 12 of the 14 patients exhibited a diffuse blastic reaction with multifocal bone resorption sites [Roudier et al., 2003]. The preferential localization of prostate cancer to the skeleton may be due to the increased bone turnover rate and the 6%–18% loss in bone mineral density that is observed in patients receiving androgen deprivation therapy [Diamond et al., 1998; Daniell et al., 2000; Stoch et al., 2001]. Data supporting this concept comes from observations of preferential dissemination of prostate cancer to the skeleton of athymic mice that exhibit increased bone turn over due to administration of parathyroid hormone [Schneider et al., 2005]. However, parathyroid hormone also induces secretion of interleukin-6 (IL-6) from osteoblasts [Feyen

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et al., 1989], which has also been implicated in the development of androgen-independent disease and will be described later.

ANDROGEN INDEPENDENT PROSTATE CANCER

Androgen deprivation therapy is the only effective systemic therapy available for patients with metastatic disease. Androgen deprivation therapy is based upon the recognition that normal and neoplastic prostate epithelial cells depend on circulating androgens for their continued growth and survival. Prospective randomized clinical trials have suggested that early therapy can increase survival [Bolla et al., 1997, 2002; Walsh, 1997; Messing et al., 1999]. Unfortunately, androgen deprivation therapy is only palliative and does not completely or permanently eliminate all malignant cell populations. This results in the predictable pattern of initial response to androgen ablation therapy indicated by a reduction in tumor volume and clinical symptoms, followed by recurrence, with the ultimate progression to androgen independence. It is believed that the progression of prostate cancer is accompanied by a shift in reliance on endocrine controls to paracrine and eventually autocrine controls and that this complex process is the result of changes, which occur at molecular levels of cellular control. Currently, the molecular mechanisms involved in the development of androgen-independent prostate cancer remain unclear. Clonal selection and adaptation have been suggested to explain progression, but these general theories have been supplanted largely by molecular concepts related to the androgen receptor (AR). Recognition of AR mutations, gene amplification, co-regulators, and cross-talk between signal transduction pathways has given rise to the possibility of studying the primary events that trigger progression.

PROSTATE-SPECIFIC ANTIGEN (PSA) AS A MARKER FOR ANDROGEN INDEPENDENT PROSTATE CANCER

PSA is a secreted protein that is abundantly expressed by prostate epithelial cells with serum levels correlated to tumor burden (Fig. 1). Serum PSA levels are routinely used by clinicians to monitor treatment responses, prognosis and progression of patients with prostate cancer. The majority of patients with advanced prostate cancer will respond to androgen deprivation therapy as measured by a reduction of clinical symptoms and tumor burden and a corresponding decrease in serum PSA to normal levels during the first 8 months of therapy [Bruchovsky et al., 1993]. However, after an initial response to therapy, higher titers of serum PSA are again observed, denoting progression to an androgen-independent state [Bruchovsky et al., 1993]. A rising titer of serum PSA after an initial response to androgen deprivation is the earliest indication of hormonal progression and is correlated with reduced survival. Once serum PSA levels are elevated in the absence of androgens, the average survival time is 2 years.

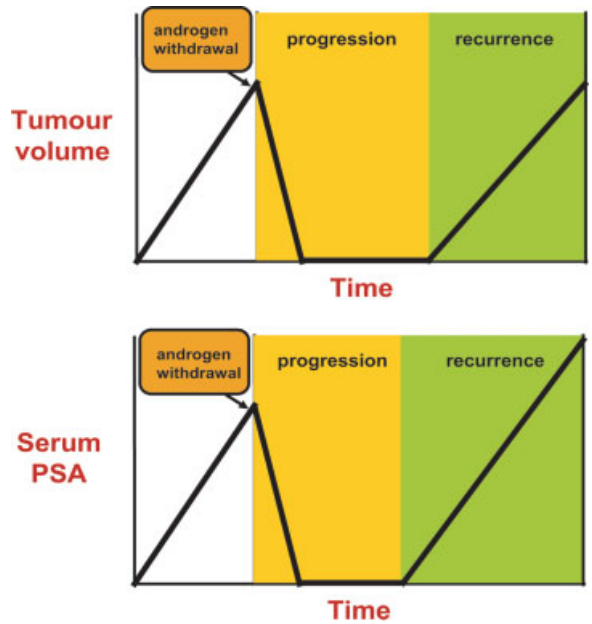

Fig. 1. In the presence of androgens, as the prostate cancer tumor volume increases there is a concomitant rise in serum PSA levels. When the patient is placed on androgen withdrawal therapy, the tumor will regress and serum PSA levels fall to a nadir. Eventually, however, even in the absence of androgens, the serum PSA levels will begin to rise again signifying biochemical failure and the onset of early tumor progression to androgen independent disease. Rises in serum PSA precede any clinical signs of progression from anywhere from several months to a few years.

The re-expression of the PSA gene in androgen-independent tumors is a transcriptional-related event as demonstrated in the LNCaP hollow fiber model and LNCaP xenograft tumor models of human prostate cancer [Sato et al., 1996; Sadar et al., 2002]. These models show the effects of continuous androgen suppression both

on serum PSA and tumor mRNA levels. Both are downregulated when testosterone is withdrawn, but later PSA protein and mRNA is constitutively upregulated despite continuing absence of testosterone indicating escape of androgen regulation of this gene. By examining the molecular mechanism of how PSA escapes regulation by androgen, clues may be revealed of how the entire prostate cancer cell escapes regulation by hormone in advanced disease. PSA is an example of an androgen-regulated gene with several well-characterized androgen response elements (AREs) to which the AR binds to initiate transcription [Riegman et al., 1991; Cleutjens et al., 1996; Schuur et al., 1996]. The re-expression of PSA suggests that the AR plays an important role in androgen independent disease. Other evidence supporting the AR as a probable factor in the hormonal progression of prostate cancer is summarized in Table I.

AR GENE AND PROTEIN STRUCTURE

Numerous review articles have been written about the basic biology of the AR thus we will only provide a brief overview here. The AR belongs to the superfamily of nuclear receptors that mediate responses to lipophilic ligands, including steroids, retinoids, vitamin D3, and thyroid hormones. The gene for the AR is located on the long arm of the X chromosome (q11–12) and consists of eight exons that encode a protein of 919 amino acids length with four structurally and functionally distinct domains (Fig. 2). The ligand-binding domain (LBD) at the C-terminus of the receptor consists of amino acids 676-919 and is the region where androgens and antiandrogens bind. The DNA-binding domain (DBD) consists of amino acids 559–624 and is essential for the binding of the receptor to androgen response elements (AREs) upstream of target genes. There is also a small hinge domain between DBD and LBD. A nuclear localization signal (NLS) spans the region between the DBD and the hinge domain. Finally, the N-terminal domain (NTD) consists of amino acids 1–558 and contains the activation function (AF) 1 region that is involved in interaction with the transcriptional machinery.

N-TERMINAL DOMAIN OF THE AR

Analysis of the amino acid sequence of the AR–NTD from a diverse range of organisms has revealed the presence of three areas of sequence conservation: amino acids 1–30, 224–258, and 500–541. The first 30 amino acids of the NTD, particularly the sequence ^{23}FxxLF^{27} and flanking residues are believed to be critical for the N/C terminal interactions (reviewed in [He et al., 2002; Steketee et al., 2002]), which contributes to the agonist-induced stabilization of the AR [He et al., 2002]. However, while the ^{23}FxxLF^{27} motif is important for optimal orientation and association of the NTD with the LBD between AR dimers, deletion of this motif did not affect dimerization affinity [Schaufele et al., 2005]. The sequence between amino acids 500 and 541, adjacent to the DBD, has been recently reported to have a negative influence on AR binding to the ARE from the first intron of the prostatic binding protein gene [Liu et al., 2003]. Residues 224–258 are within the AF1 transactivation domain of the AR. This sequence has been

TABLE I. Evidence Supporting a Role for the AR in Hormonal Progression of Prostate Cancer

Evidence	References
The AR is expressed in the nuclei of the majority of hormone refractory tumors	[van der Kwast et al., 1991; Hobisch et al., 1995; Kim et al., 2002]
Mutations in the AR can result in hypersensitivity to castrate levels of androgens or in activation by non-androgenic steroids	[Gottlieb et al., 1999]
Amplification of the AR gene has been detected in 20% to 30% of androgen independent tumors	[Visakorpi et al., 1995]
Genes normally regulated by androgens such as prostate-specific antigen (PSA) are re-expressed in androgen-independent disease	[Gregory et al., 1998]
The timing and sequence of use of the family of anti-androgens may prolong the time to androgen independence	[Bruchovsky et al., 2000]
Ligand-independent activation of the AR has been shown to occur in prostate cancer cells maintained in monolayer	[Culig et al., 1994; Nazareth and Weigel, 1996; Sadar, 1999; Ueda et al., 2002a,b]
The AR was shown to be necessary for the proliferation of androgen independent prostate cancer cells	[Zegarra-Moro et al., 2002]
The low levels of androgen remaining in clinical tissues from castrated men may be sufficient to mediate biological activity	[Geller et al., 1979; Mohler et al., 2004]
Increased AR expression is associated with the development of resistance to antiandrogen therapy	[Chen et al., 2004]

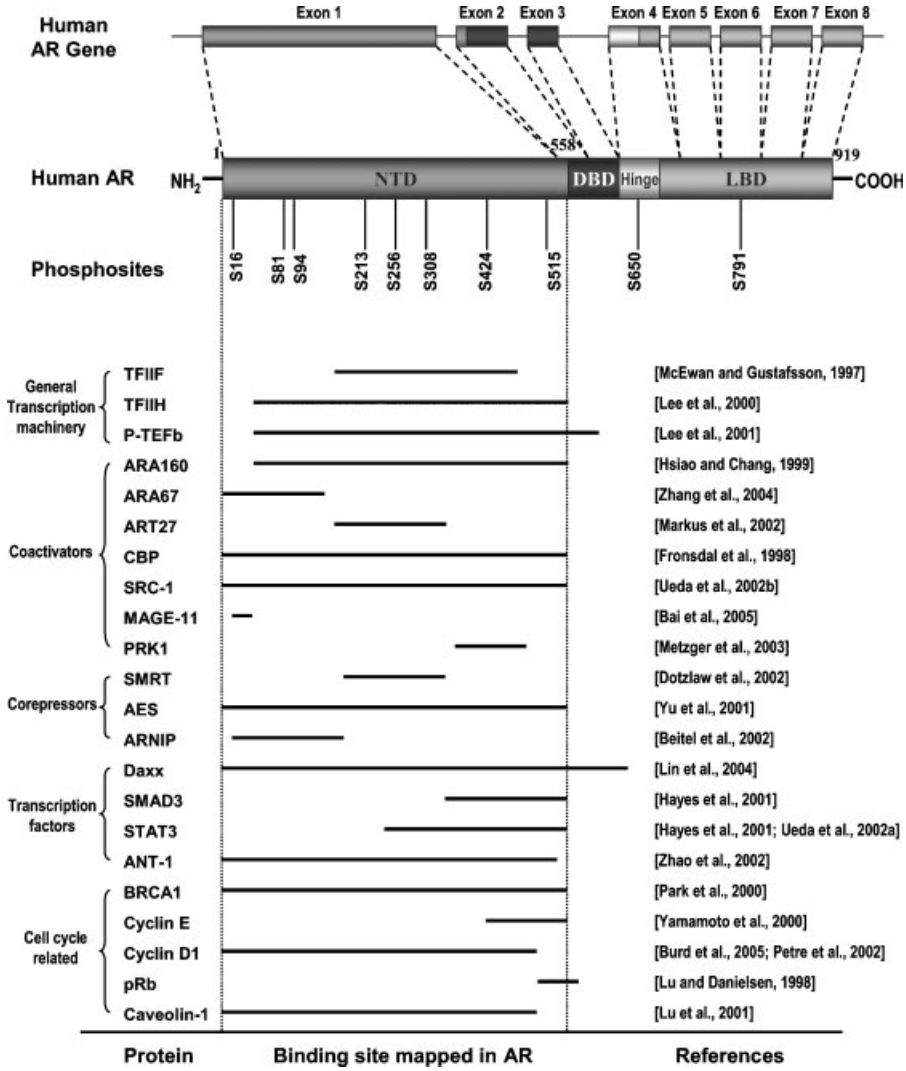

Fig. 2. AR gene organization and domain structure of the protein. The AR gene consists of eight exons that give rise to the characteristic domain structure of the receptor protein. NTD, N-terminal domain; LBD, ligand-binding domain; DBD, DNA-binding domain. Known sites of phosphorylation (P) are shown. Proteins binding to AR NTD and their binding sites mapped in AR are shown. See text for details.

highly conserved in the AR from fish to primates, showing 60% homology over a stretch of 35 amino acids, and is characterized by the presence of several functionally important, bulky hydrophobic amino acids [Betney and McEwan, 2003]. Unlike other members of the nuclear receptor superfamily, the main determinant for receptor-dependent transcriptional activation of AR resides within the AF1 domain (amino acids 142–485) [Simental et al., 1991; Jenster et al., 1995]. When fused to the LexA–DBD, this region retained at least 70% of the activity of the full-length AR–NTD; importantly, a double point mutation that significantly reduced the activity of the full-length AR had an identical effect on the isolated AR–AF1 domain [Betney and McEwan, 2003]. In the past, the AF-1 region of the AR was called ligand-independent based on experiments demonstrating that the AR NTD could activate a reporter gene in the absence of the LBD to a level comparable to that achieved with the full-length receptor [Jenster et al., 1995]. To avoid confusion, this was later termed "constitutive activation" of the AR NTD because under the same cell conditions, the full-length receptor is not activated [Ueda et al., 2002b]. Constitutive activation of the AR NTD can therefore be distinguished from conditions where activation of the AR NTD by IL-6 and other compounds,

which activate the full-length AR in the absence of androgens, is termed as ligand-independent activation [Ueda et al., 2002b].

ACTIVATION OF THE AR

The process of ligand-induced transformation of the AR is not completely understood, although it is known that the unliganded AR exists predominantly in the cytoplasm in an unfolded state [Schaufele et al., 2005]. Upon ligand binding: (1) heatshock proteins are dissociated; (2) the AR translocates to the nucleus; (3) the AR conformation changes; (4) the AR forms homodimers; (5) post-translational modifications of the AR occur, such as phosphorylation and acetylation; (6) there are changes in protein-protein interactions with the AR; and (7) the AR binds to AREs on the DNA. The transcriptional activity of the AR is enhanced by the recruitment of coactivators, and release of corepressors [Collingwood et al., 1999]. In the presence of ligand, the AR is activated to stimulate or repress androgen-regulated genes. However, the AR is also transformed in the absence of androgen in prostate cells by growth factors, such as IL-6, and stimulation of protein kinase pathways such as MAPK and PKA [Culig et al., 1994, 1997a; Ikonen et al., 1994; Nazareth and Weigel, 1996; Hobisch et al., 1998; Sadar, 1999; Sadar and Gleave, 2000; Ueda et al., 2002a,b]. The mechanism of ligand-independent activation of AR has not been clarified but involves its NTD [Sadar, 1999] and may bypass one of the above mechanisms of ligand-dependent transformation. Of these mechanisms, phosphorylation is the most generally accepted model for ligand-independent activation of the progesterone, estrogen, and retinoic acid receptors. This may involve changes in phosphorylation of the AR itself, or changes in the phosphorylation of an interacting protein.

LIGAND-INDEPENDENT ACTIVATION OF THE AR BY MULTIPLE PROTEIN KINASE PATHWAYS

Enhanced phosphorylation of steroid receptors and/or associated proteins is concomitant with increases in transcriptional activity of the receptor upon binding of hormone [Denner et al., 1990; Bodwell et al., 1991; Orti et al., 1992]. The AR NTD contains a number of putative phosphorylation sites for serine-proline-directed kinase, DNA-dependent kinase, protein kinase C, casein kinase I and II, PKA, MAPK, Akt, calmodulin kinase II, and tyrosine kinases.

Several phosphorylation sites have been identified in the AR (Fig. 2). The first identified phosphosites, Ser-81, Ser-94 and Ser-650, were found by mutagenesis analyses in combination with SDS–PAGE [Jenster et al., 1994; Zhou et al., 1995]. Ser-308 was the first phosphosite identified by mutagenesis and mass spectrometry [Zhu et al., 2001]. Ser-16, Ser-81, Ser-94, Ser-256, Ser-308, Ser-424, and Ser-650 were all identified and confirmed as phosphosites by mutagenesis, peptide mapping, and mass spectrometry [Gioeli et al., 2002]. Cell-free in vitro phosphorylation reaction studies on AR mutants revealed that Ser-213, Ser-515, and Ser-791 are phosphosites [Yeh et al., 1999; Wen et al., 2000; Lin et al., 2001]. It is striking that the majority of identified phosphorylation sites map to the AR–NTD, particularly the AF-1 region, suggesting that these modifications may directly modulate receptor-dependent transactivation. Possible mechanisms could involve altering protein–protein interactions and/or alterations in protein structure and stability. It is currently unclear whether differing functions are linked to different phosphorylation sites or whether phosphorylation at certain sites is pluripotent. However, as phosphorylation of the receptor can be induced by many mechanisms, it is likely that AR phosphorylation is a multistage process.

When AR is first synthesized it is not phosphorylated but after about 15 min, even in the absence of a ligand, it becomes phosphorylated at some sites. Ser-94 is constitutively phosphorylated and in response to androgen the AR becomes hyperphosphorylated on serines 16, 81, 256, 308, 424, and 650 [Gioeli et al., 2002]. This phosphorylation is thought to protect the AR from proteolytic degradation and stabilize AR homodimers [van Laar et al., 1990]. In addition to the protective and stabilizing role of AR phosphorylation, evidence is now emerging that phosphorylation of the AR at specific sites can influence transactivation [Lee and Chang, 2003]. AR transcriptional activity also correlates strongly with phosphorylation of specific serine residues [Rochette-Egly, 2003].

MAPK

Mitogen-activated protein kinase (MAPK) is elevated in recurrent prostate cancer [Gioeli

et al., 1999] and required for both ligand-dependent and ligand-independent activation of the AR [Ueda et al., 2002a]. Androgen, IL-6 and stimulation of the PKA pathway all increase MAPK phosphorylation [Ueda et al., 2002a,b]. Phosphorylation of the AR by MAPK positively modulates the expression of AR target genes, helps the recruitment of AR cofactors, and increases prostate cancer cell growth [Rochette-Egly, 2003]. In LNCaP cells, phosphorylation of AR at Ser-515 by MAPK results in hyper-sensitization to low levels of the synthetic androgen R1881 [Bakin et al., 2003]. It therefore may be possible that during androgen blockade, AR phosphorylation by MAPK induces hypersensitivity to castrate levels of androgens to result in increased recruitment of AR cofactors and AR transcription activity. The common requirement of MAPK for both ligand-dependent and ligand-independent activation of the AR indicates that inhibition of MAPK activity may constitute a general mechanism for antagonizing AR function in prostate cancer cells [Ueda et al., 2002a].

AKT

Phosphorylation of AR by Akt has shown contradictory results. Some suggest that Akt may play a similar role to MAPK in the development of androgen-independent disease [Liao et al., 2003b] since Akt may phosphorylate the AR at Ser-213 and Ser-791 and modulate its transcriptional activity in prostate cancer cell lines [Ghosh et al., 2003]. During androgen ablation or antiandrogen treatment, LNCaP cells undergo growth arrest and apoptosis, and up-regulation of Akt activity appears to compensate for this [Gao et al., 2003]. Androgen-independent LNCaP cells have high basal Akt activity (>20 times higher than sensitive cells) [Lin et al., 2003], suggesting that removing androgens by androgen ablation therapy may result in increased activation of the PI3K pathway and promote the development of androgen independence [Ghosh et al., 2003]. This hypothesis is supported by data showing that up-regulating the PI3K cascade on loss of androgen signaling contributes to the failure of androgen ablation therapy [Murillo et al., 2001]. However, other studies showed that the pattern of activated Akt correlates with the pattern of phosphorylation of AR at S213, which may result in suppression of AR-mediated transcription [Taneja et al., 2005]. Even in the same cell line, Akt might differentially regulate AR activity at different cell passage number. In LNCaP cells at lower passage number, Akt suppressed AR activity while increased activation of the AR was reported at higher passage number [Lin et al., 2003]. The phenotype of LNCaP cells changes with continuous propagation in culture and therefore the use of different passage number may be one reason for divergent results observed on regulation of AR activity by Akt.

PKC

The AR contains two consensus sites for PKC phosphorylation at Ser-81 and Ser-650 [Gioeli et al., 2002]. Activation of PKC using phorbol 12-myristate 13-acetate (PMA) increases the phosphorylation of AR at Ser-650, but has no effect on Ser81 [Gioeli et al., 2002]. A role for Ser-650 in AR transactivation was suggested due to a subtle decrease in AR transactivation of the mouse mammary tumor virus promoter when a S650A mutant of AR was used with suboptimal levels of steroid [Zhou et al., 1995]. However, no effect was observed with the PSA-luciferase reporter with double mutants of these phosphorylation sites S81A/S650A or S81D/S650D compared to wild-type AR in CV-1 cells in response to the synthetic androgen R1881 [Gioeli et al., 2002]. It is unknown whether the lack of any effect in the latter study is: (1) specific to CV-1 cells; (2) due to the requirement for at least four mutations to be present before changes can be measured using these approaches, as shown be others; (3) due to use of transient instead of stable expression of proteins; and/or (4) due to use of a complex promoter instead of a simple reporter, which was required to measure an effect with mutated phosphorylation sites of the glucocorticoid receptor (GR) [Webster et al., 1997]. Regardless, there is little clinical evidence at present to support a role for PKC in the development of androgen independent disease.

PKA

Cross-talk between the AR and PKA signal transduction pathways occurs in androgen-depleted human prostate cancer cells maintained in culture [Nazareth and Weigel, 1996; Culig et al., 1997a,b; Sadar, 1999; Kasbohm et al., 2005]. These studies have shown that anti-androgens can block PKA induction of PSA mRNA [Sadar, 1999] and androgen-responsive

reporters [Nazareth and Weigel, 1996; Culig et al., 1997a; Sadar, 1999]. Further evidence supporting ligand-independent activation of the AR through the PKA pathway includes increased AR-ARE complex formation with nuclear extracts from cells exposed to activators of PKA. Interestingly, more AR-ARE complex formation occurred in the presence of nuclear extracts from forskolin-treated cells than from androgen-treated cells, even though the nuclear levels of AR were approximately 10-fold higher in the androgen-treated cells [Sadar, 1999]. This suggests that the PKA-transformed AR may have a greater affinity for the PSA-ARE than the receptor activated by androgen. Such a theory is supported by the fact that the AR-NTD is activated by PKA in LNCaP cells [Sadar, 1999] and this region of the AR contributes to the stability of the receptor-DNA complex [Kallio et al., 1994]. To date there have been no studies examining the in vivo effects of activation of the PKA pathway on phosphorylation of the AR using appropriate levels of compounds. Although Gioeli found increased phosphorylation of the AR at Ser-650 in response to 50 μM forskolin, a PKA activator [Gioeli et al., 2002], this concentration of forskolin inhibits activation of the AR in non-transfected cells [Blok et al., 1998; Sadar, 1999]. Forskolin at 1 μM concentration is optimal to induce PSA mRNA, nuclear translocation, activation of the AR and its DNA-binding activity, with 50 μM inhibiting AR in non-transfected LNCaP cells [Blok et al., 1998; Sadar, 1999]. Thus no studies have been reported that examine the phosphorylation of AR in response to compounds, at appropriate concentrations, that activate the AR in the absence of androgens.

ACTIVATION OF THE AR BY THE IL-6 SIGNALLING PATHWAY

IL-6 is a cytokine that activates target genes involved in differentiation, survival, apoptosis, and proliferation. Signal transduction of IL-6 involves the activation of JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. Another major signaling pathway for IL-6 is the MAPK cascade (reviewed in [Heinrich et al., 2003]). There is considerable clinical interest in the IL-6 signal pathway in the progression of prostate cancer for a number of reasons summarized in Table II. Most importantly is its role in bone, the predominant site of prostate cancer metastasis. IL-6 is synthesized by osteoblasts that promotes osteoblastogenesis and bone formation [Taguchi et al., 1998]. Interactions between prostate

TABLE II. Evidence Supporting a role for the IL-6 in Advanced Prostate Cancer

Evidence	References
Prostate cancer predominately metastasizes to bones that express IL-6	[Haq et al., 1992; Hobisch et al., 1998]
Epithelial cells from normal, hyperplasia, and carcinoma prostate tissue also secrete IL-6 in culture media	[Twillie et al., 1995]
The IL-6 receptor is expressed in normal prostate, high-grade prostatic intraepithelial neoplasia, and cancer	[Siegsmund et al., 1994; Hobisch et al., 2000]
IL-6 is elevated in the sera of patients with metastatic prostate cancer and hormone-refractory disease	[Twillie et al., 1995; Adler et al., 1999; Drachenberg et al., 1999; Shariat et al., 2001]
Elevated serum levels of IL-6 are associated with poor prognosis for men with prostate cancer	[Adler et al., 1999; Nakashima et al., 2000; Wise et al., 2000]
IL-6 potentiates neuroendocrine differentiation of LNCaP cells, which is a phenotype associated with poor prognosis	[di Sant'Agnese, 1992; Qiu et al., 1998]
An increase in proliferation of prostate cancer cells has been shown in response to IL-6	[Okamoto et al., 1997; Qiu et al., 1998; Lou et al., 2000; Giri et al., 2001; Smith and Keller, 2001; Ueda et al., 2002a]
Neutralizing antibody inhibits the proliferation of PC-3 and DU145 prostate cancer cells that endogenously express IL-6 and inhibits proliferation and activation of the AR in LNCaP cells in response to bone derived factors	[Borsellino et al., 1995, 1999; Chung et al., 1999; Blaszczyk et al., 2004]
The IL-6R is expressed in Du145, PC3, and LNCaP cells, which also express gp130 and secrete soluble IL-6Rα	[Siegall et al., 1990; Okamoto et al., 1997; Chung et al., 1999]
STAT3 signaling downstream of IL-6R activation was shown to be important for the progression of prostate cancer cells and for transformation of cells. Consistent with these observations is the finding of elevated constitutive STAT3 activity in 82% (37/45) of primary prostate cancer tumors and small interfering RNA (siRNA) to STAT3 suppresses growth and induces apoptosis of human prostate cancer cells while also inhibiting PSA gene expression in these cells	[Ni et al., 2000]
IL-6 activates the AR thru its NTD in the absence of androgens in prostate cancer cells to increase its transcriptional activity and induce the expression of androgen regulated genes such as PSA	[Ueda et al., 2002a]

cancer cells and osteoblasts increase the local concentrations of IL-6 [Garcia-Moreno et al., 2002]. Androgens inhibit the expression of IL-6 in osteoblasts [Bellido et al., 1995], which express AR [Colvard et al., 1989]. Overexpression of IL-6 renders LNCaP cells more resistant to apoptosis and promotes androgen-independent growth in vitro and in vivo [Lee et al., 2003, 2004; Pu et al., 2004]. Increased proliferation possibly involving ligand-independent activation of the AR in response to osteoblast-derived factors was recently shown to be dependent upon IL-6 [Blaszczyk et al., 2004]. IL-6 produced by osteoblasts may therefore encourage osseous metastasis through activation of the AR in prostate cancer cells to enhance survival and increase proliferation [Ueda et al., 2002a; Blaszczyk et al., 2004]. Thus androgen deprivation therapy would elevate IL-6 production in the bone to provide an environment that may stimulate androgen independent growth of prostate cancer cells possibly by activating the AR NTD to cause re-expression of genes normally regulated by androgens that are important for proliferation [Ueda et al., 2002a].

AR-NTD AS THE TARGET OF IL-6 SIGNAL PATHWAYS

Both ligand-independent and synergistic effects of IL-6 on AR activity are observed in prostate cancer cells [Hobisch et al., 1998]. Functional JAKs/STAT3 and MAPK pathways are required for activation of the AR NTD by IL-6 [Hobisch et al., 1998; Chen et al., 2000]. Immunoprecipitation and transactivation studies showed a direct interaction between amino acids 234–558 of the AR-NTD and STAT3 following IL-6 treatment of LNCaP cells [Ueda et al., 2002a]. Inhibitors to MAPK and JAK decreased the IL-6-induced phosphorylation of MAPK and activation of the AR-NTD, demonstrating that activation of the human AR-NTD by IL-6 is dependent upon MAPK and STAT3 signal transduction pathways in LNCaP prostate cancer cells [Ueda et al., 2002a]. The steroid receptor coactivator, SRC-1, interacts with the AR NTD to increase AR transcriptional activity [Ueda et al., 2002b]. Protein–protein interaction between endogenous AR and SRC-1 is dependent upon IL-6 but independent of MAPK activity. However, phosphorylation of SRC-1 by MAPK is required for optimal ligand-independent activation of the AR by IL-6 [Ueda et al., 2002b]. Ligand-independent activation of the AR did not occur by a mechanism of overexpression of either solely wild-type SRC-1 or mutant SRC-1 that mimics its phosphorylated form. The co-activator CREB-binding protein (CBP) is involved downstream of the MAPK pathway during transactivation of the AR by IL-6 [Debes et al., 2002]. Both the AR NTD and LBD can interact with CBP [Fronsdal et al., 1998]. Phosphorylation of SRC-1 by MAPK is required for optimal PR-dependent transcription and for functional cooperation with CBP [Rowan et al., 2000a]. Thus, although SRC-1 interacts with the AR NTD in response to IL-6, the inhibitory effects of blocking MAPK phosphorylation on the activity of the AR NTD may be due to preventing the interaction of CBP with SRC-1 (Fig. 3). The clinical relevance for such a mechanism involving IL-6, MAPK and SRC-1 in activating the AR in prostate cancer is probable since patients with androgen-independent prostate cancer have elevated levels of IL-6 [Twillie et al., 1995; Adler et al., 1999], SRC-1 [Fujimoto

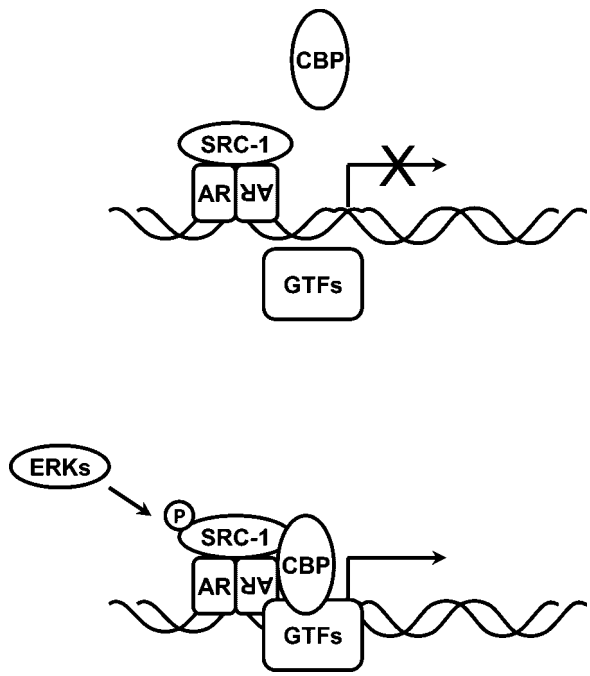

Fig. 3. Model for MAPK phosphorylation of SRC-1 and AR activation in LNCaP cells. In the absence or reduction in phosphorylation, SRC-1 cannot form a fully functional complex with CBP, and transcriptional synergism is lost (**top**). When SRC-1 is phosphorylated by ERK-1/2 (MAPK) in response to IL-6, PKA, or androgen, it is then able to form a functional complex at the promoter that results in maximal gene transcription (**bottom**). GTFs, general transcription factors; P, phosphorylation.

et al., 2001; Gregory et al., 2001], and MAPK activity [Gioeli et al., 1999].

AR PHOSPHORYLATION IS CELL CYCLE RELATED

Androgens are potent mitogens that shorten the length of G_1/G_0, accelerate entry into S phase by affecting expression or activity of cyclins and CDKs [Gregory et al., 2001]. The transcriptional activity of the murine AR (mAR) varies throughout the cell cycle in mouse L929 cells [Martinez and Danielsen, 2002]. The mAR is transcriptionally active in G_0 and S phase, but loses over 90% of its activity during G_1/S. This effect is specific to the AR. Levels of mAR protein are decreased at G_1/S, but this cannot explain the lack of transcriptional activity. The idea that different transactivation of AR during the cell cycle is phosphorylation related is supported by the evidence from other nuclear receptors. The PR is a substrate of cyclin A/Cdk2, a late G_1- and S-phase kinase, and Cdk2 is important for PR activity [Knotts et al., 2001; Narayanan et al., 2005a]. Phosphorylation of PR at Ser-162 and Ser-294 is impaired in the G_2/M phase while phosphorylation of Ser-190 remains comparable to levels seen in other phases of the cell cycle [Narayanan et al., 2005b]. Phosphorylation of Ser-294 is dependent upon hormone. Importantly, nucleocytoplasmic shuttling of PR also varies as a function of the cell cycle; being most prominent in the nucleus in S phase [Narayanan et al., 2005b]. Similar results have been found in the GR. GR is transcriptionally inactive in G_2/M [Hsu et al., 1992] and importantly, the pattern of GR phosphorylation changes during the cell cycle [Hu et al., 1994]. Data supporting the role of cell cycle in phosphorylation of the AR can be drawn from studies showing phosphorylation of Ser-213 of the AR in differentiated cells lining the lumen of the urogenital sinus, but not in rapidly dividing, Ki67 positive cells within the developing prostate or stromal tissue [Taneja et al., 2005]. AR Ser-213 is phosphorylated at high concentrations of androgen (10 nM) but not at lower concentrations [Taneja et al., 2005]. This is consistent with 10 nM androgen causing cell cycle arrest and while the lower concentration of androgen promote proliferation. To date there are no reports that have examined the AR phosphorylation or nucleocytoplasmic shuttling throughout the cell cycle in spite of the evidence from other related steroid receptors that these important events vary as a function of cell cycle [Narayanan et al., 2005b]. Since the AR NTD domain contains the majority of identified phosphorylation sites and interacts with a number of proteins involved in the regulation of cell cycle (see below) it seems probable that these events will vary as a function of cell cycle and play an important role in the proliferation of androgen independent prostate cancer.

STRUCTURAL BASIS FOR COREGULATOR RECRUITMENT

Expression of androgen-regulated genes is affected by co-regulators that influence function of the AR, including ligand selectivity and DNA-binding capacity [Heinlein and Chang, 2002]. Co-regulators modify the transcriptional activity of the AR, which could play a role in the progression of prostate cancer. At the promoter of target genes, co-regulators participate in DNA modification, either directly through modification of histones or indirectly by the recruitment of chromatin-modifying complexes. Co-regulators also function in the recruitment of the basal transcriptional machinery. Aberrant co-regulator activity due to mutation or altered expression levels may be a contributing factor in the progression of diseases related to AR activity, such as prostate cancer. Importantly, the activity of coactivators can also be regulated by phosphorylation [Rowan et al., 2000a,b; Wu et al., 2002]. Such post-translational modifications are thought to be important for cell/tissue type- and promoter-specificity [Wu et al., 2005]. The AR demonstrates distinct differences in its interaction with co-regulators from other steroid receptors due to differences in the functional interaction between AR domains, possibly resulting in alterations in the dynamic interactions between co-regulator complexes [Heinlein and Chang, 2002].

Ligand-induced conformational changes of the AR provide the structural basis for the recruitment of cofactor proteins and transcriptional machinery, which is required for the assembly of AR-mediated transcription complexes [Shang et al., 2002]. A functional AF2 region is believed to be crucial for coactivator recruitment, because the so-called nuclear receptor box "LxxLL" motif from the nuclear-receptor-interacting domain (NID) of

co-activators specifically binds to this surface [Bourguet et al., 2000]. However, similar motifs from the AR-NTD, ^{23}FxxLF^{27}, and ^{433}WxxLF^{437}, can also interact with this region [He et al., 2002]. Therefore, both interactions could compete for the AF2 region upon agonist binding. Despite the overall similarity in peptide binding modes, ligand-bound LBD prefers the binding of the FxxLF motif in the NTD to that of the LxxLL motif in co-regulators, suggesting that N/C interaction is preferred over co-activator recruitment in ligand-bound AR [He et al., 2002]. The association of the AR-NTD and LBD precludes other protein factors from binding to the coactivator-binding pocket in LBD, and thus co-activator proteins responsible for the transcriptional activity of the AR must be recruited to an alternative surface, possibly within the NTD. Indeed, transcriptional activation by AR at certain promoters does not require cofactor binding to the LBD [Simental et al., 1991; He et al., 1999]. This is consistent with the observation that the AR-LBD alone is a poor transcriptional activator, and that the AR-NTD exhibits activity similar to the full-length AR [Chang and McDonnell, 2005]. Promoter-specificity has been observed between ligand activated AR and ligand-independent activated AR [Sadar, 1999; Sadar and Gleave, 2000]. This suggests that ligand-independent activation of the AR may cause the AR to adopt a different conformation to that assumed by the ligand-bound AR thereby resulting in the exposure of different regions of the AR for alternative protein–protein interactions. Such differences in conformation would imply differences in the subset of co-activators recruited to the promoter between AR activated by ligand and the AR activated by non-androgenic pathways.

AR COREGULATOR RECRUITMENT

AR co-activators enhance transactivation of AR several fold and therefore potentially increase the risk of prostate cancer. In vitro studies have shown that certain AR co-activators, such as: SRC-1; AR associated (ARA) 54, 55, 70, 160; p160; breast cancer susceptibility genes (BRCA1 and 2); and AIB1 (amplified in breast cancer 1), can enhance AR transcriptional activity several-fold in prostate cancer cells [Heinlein and Chang, 2002]. See http://ww2.mcgill.ca/androgendb/Arinteract.pdf for a list of proteins known to interact with the AR. Note that few proteins (SRC-1, Daxx, Dax1, SMRT, NCoR) have been identified from endogenous complexes with the AR in prostate cells [Cheng et al., 2002; Ueda et al., 2002b; Agoulnik et al., 2003; Liao et al., 2003a; Lin et al., 2004]. The importance of validating endogenous complexes in relevant systems is based upon: (1) protein–protein interactions are dependent upon concentration, and thus overexpression may lead to false-positives; (2) over-expression or forced expression of proteins may cause aberrant cellular localization and/or inappropriate timing of expression if the expression of proteins are dependent upon the cell cycle phase; (3) the AR NTD is not correctly folded in vitro [Reid et al., 2002] and thus in vitro interaction assays, such as the glutathione S-transductase pull-down assay, may yield erroneous results; and/or (4) protein modifications required for interactions may vary in different cells. These points are especially important when investigating protein–protein interactions of the AR activated by non-androgenic pathways involving signal transduction pathways that may have cell-specific effects. Studies examining interaction between endogenous SRC-1 and AR in prostate cancer cells revealed a number of discrepancies with results obtained using yeast or other non-prostate cell lines combined with transfections [Ueda et al., 2002b].

Proteins Interacting With the AR NTD

Proteins revealed to interact with the AR NTD include: proteins involved in the basal transcriptional machinery (TFIIF, TFIIH, P-TEFb); coactivators (SRC-1, ARA67, ARA160, ART27, CBP, MAGE-11, PRK1); co-repressors (SMRT, AES); transcription factors (SMAD3, Daxx, STAT3, ANT-1); and proteins that play a role cell cycle (BRCA1, cyclin E, cyclin D1, pRB, caveolin-1, ARNIP) (Fig. 2).

General transcriptional machinery. Based upon the fact that the AR NTD contains the AF-1 region perhaps it is not surprising that it interacts with the general transcriptional machinery such as TFIIF [McEwan and Gustafsson, 1997]. Co-transfection of cdk activating kinase (CAK), the kinase moiety of TFIIH, enhanced AR-mediated transcription in a ligand-dependent manner in human prostate cancer PC-3 and LNCaP cells, and in a ligand-independent manner in human

prostate cancer DU145 cells. CAK is also involved cell cycle. Detailed interaction studies further revealed that the AR NTD (amino acids 141–485) interacting with CAK was essential for the CAK-induced AR transactivation [Lee et al., 2000]. P-TEFb interacts with amino acids 38–634 of AR and enhances the elongation stage of transcription by phosphorylation of the large subunit of RNA PoIII enzyme [Lee et al., 2001].

Co-activators. In addition to SRC-1, a number of co-activators of the AR that interact with the NTD have been identified. These include CBP, which physically interacts with the AR in vitro as shown in glutathione S-transferase pulldown assays [Fronsdal et al., 1998]. CBP bridges the interaction between the AR and CREB in response to forskolin [Kim et al., 2005]. AR and ARA160/TMF, a coactivator for AR-mediated transactivation in human prostate cancer, interacts directly with the AR NTD (amino acids 38–566). This interaction is significantly enhanced by androgen [Hsiao and Chang, 1999]. ART-27 is a novel 157 amino acid protein identified in a yeast two hybrid screen, and it has been shown to interact with amino acids 153–366 in the AR NTD [Markus et al., 2002]. The X chromosome-linked melanoma antigen gene product MAGE-11 is an AR coregulator that specifically binds the AR NTD FXXLF motif. Binding of MAGE-11 to the AR FXXLF alpha-helical region stabilizes the ligand-free AR and, in the presence of an agonist, increases exposure of AF2 to the recruitment and activation by the SRC/p160 coactivators [Bai et al., 2005]. ARA67/PAT1 is an AR NTD (amino acids 1–140) interacting protein [Zhang et al., 2004]. RhoA effector protein kinase C-related kinase PRK1 can enhance the transcriptional activation of the AR. AR and PRK1 interact both in vivo and in vitro. The transactivation unit 5 (TAU-5, amino acids 360-485) located in the NTD of AR suffices for activation by PRK1 [Metzger et al., 2003].

Repressors. The binding site for the corepressor SMRT has recently been mapped to the same amino acids as ART-27 [Dotzlaw et al., 2002]. Thus, the binding sites for ART-27 and SMRT within the AR-transactivation domain potentially overlap with each other and with TFIIF, and it will be interesting to determine whether these proteins compete for receptor binding. AES is a selective repressor of ligand-dependent AR-mediated transcription and acts by directly interacting with AR-NTD and targets the basal transcription machinery [Yu et al., 2001]. ARNIP interacts with amino acids 11–172 of AR to inhibit AR N/C-terminal interaction [Beitel et al., 2002]. ARNIP is involved in ubiquitin-mediated proteosomal degradation of p53 thereby influencing cell cycle.

Transcription factors. Interactions between the AR and other transcription factors provides a mechanism of cross-talk between different signal transduction pathways. Interactions between the AR NTD (amino acids 330–563) and SMAD3 provide a mechanism of cross-talk between the TGF-β and AR signal transduction pathways [Hayes et al., 2001]. As already discussed, STAT3 binding to the AR NTD links these two pathways [Hayes et al., 2001; Ueda et al., 2002a]. Daxx is a transcription factor thought to be involved in apoptosis and functions as a negative AR coregulator through direct protein–protein interactions with both the NTD and the DBD of the AR [Lin et al., 2004]. AR N-terminal domain transactivating protein-1 (ANT-1), a p102 U2 small nuclear ribonucleoprotein particle binding protein, enhances ligand-independent autonomous AF-1 transactivation function of AR [Zhao et al., 2002].

Cell cycle. BRCA1 plays a role in DNA repair and cell cycle arrest. BRCA1 enhances AR-dependent transactivation of a probasin-derived reporter gene by a mechanism mediated through the AF-1 in the AR NTD [Park et al., 2000]. Caveolin-1 couples integrins to the Ras/Erk to promote cell cycle progression and can potentiate ligand-dependent AR activation by interaction with both the AR NTD and LBD [Lu et al., 2001]. Cyclin D1 plays an important role in cell cycle and functions as a co-repressor of the AR NTD to inhibit ligand-dependent AR activation [Petre et al., 2002; Burd et al., 2005]. Other regulators of the cell cycle that interact with the AR NTD include cyclin E, which was shown to increase the transactivation activity of the human AR in the presence of its ligand [Yamamoto et al., 2000], and pRb, which increased transcriptional activity of the AR [Lu and Danielsen, 1998]. Based upon studies performed with other steroid receptors, it is highly probable that these interactions are specific to particular phases of the cell cycle and may depend upon AR phosphorylation or

nucleocytoplasmic shuttling that may also vary as a function of cell cycle.

SUMMARY AND PERSPECTIVES

Currently, androgen ablation therapy is used for the clinical management of advanced systemic disease that usually involves osseous metastases. This form of therapy is only palliative and once the disease becomes androgen independent there are no effective treatments that enhance survival by more than several months. One such mechanism thought to be involved in the development of androgen independent disease is ligand-independent activation of the AR through the IL-6 and/or

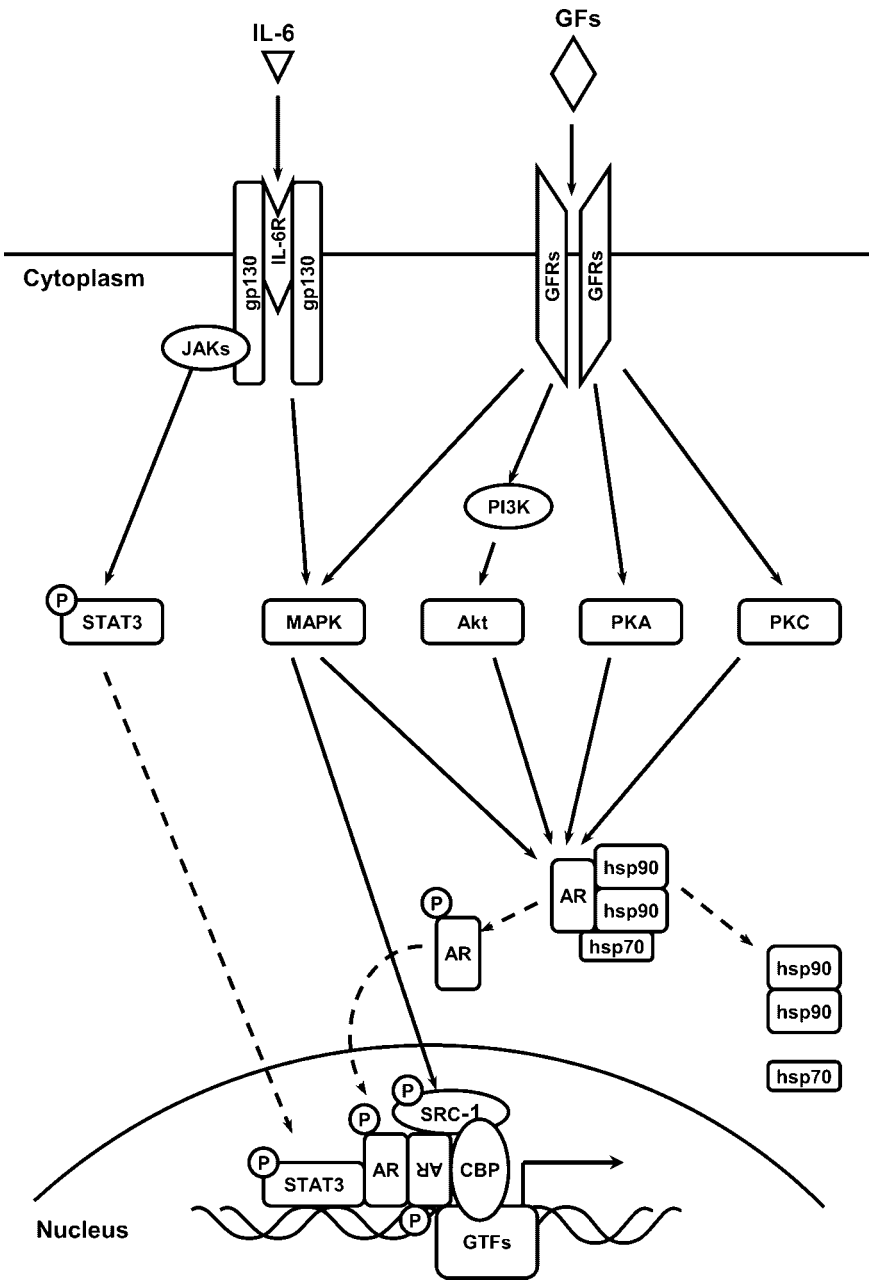

Fig. 4. Ligand-independent activation of the AR. Upon phosphorylated by the protein kinases, the AR undergoes conformational changes involving the dissociation of heat shock proteins, N/C-terminal interaction and receptor stabilization. The AR translocates to the nucleus where dimerization and DNA binding to regulatory androgen response elements occurs. IL-6, not only activates SRC-1 through MAPK to recruit CBP, but also activates STAT3 which serves as a coactivator of AR. Hsp, heat shock protein; GTFs, general transcription factors; P, phosphorylation; GFs, growth factors; GFRs, growth factor receptors. ----, translocation; →, stimulation.

protein kinase pathways that target the AR NTD. However, the mechanism of how the AR NTD becomes activated by these pathways is unknown. In order for the AR to be transcriptionally active, the unliganded AR that is usually found unfolded in the cytoplasm would have to translocate to the nucleus and assume a conformation compatible with transcriptional activity. This would require the loss of heat shock proteins, post-translational modifications, DNA-binding activity, and interaction with the general transcriptional machinery and accessory proteins essential for transcriptional activity (Fig. 4). Thus studies investigating protein–protein interactions and post-translational modifications of the AR activated in the absence of ligand should yield potential therapeutic targets for the treatment of prostate cancer. Identification of the AR NTD as the site of activation by alterative pathways provides a novel therapeutic target for the development of new drugs that may delay or avert the progression of prostate cancer to the terminal, hormone refractory stage.

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