Phosphorylation of HSP90 by protein kinase A is essential for the nuclear translocation of androgen receptor

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Running Title: Role of protein kinase A in androgen signaling

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Abstract
The androgen receptor (AR) is often activated in prostate cancer patients undergoing androgen ablative therapy because of the activation of cellular pathways that stimulate the AR despite low androgen levels. In many of these tumors the cAMP-dependent protein kinase A (PKA) pathway is activated. Previous studies have shown that PKA can synergize with low levels of androgen to enhance androgen signaling and consequent cell proliferation, leading to castration resistant prostate cancer. However, the mechanism by which PKA causes AR stimulation in the presence of low/no androgen is not established yet. Here, using immunofluorescence immunoblotting assays, co-immunoprecipitation, siRNA-mediated gene silencing, and reporter gene assays we demonstrate that PKA activation is necessary for the phosphorylation of heat shock protein (HSP90) that binds to un-liganded AR in the cytoplasm, restricting its entry into the nucleus. We also found that PKA-mediated phosphorylation of the Thr-89 residue in HSP90 releases AR from HSP90, enabling AR binding to HSP27 and its migration into the nucleus. Substitution of the Thr-89 in HSP90 prevented its phosphorylation by PKA and significantly reduced AR transactivation and cellular proliferation. We further observed that the transcription of AR target genes such as prostate specific antigen (PSA) is also lowered in the HSP90 Thr-89 variant. These results suggest that using a small-molecule inhibitor against the
HSP90 Thr-89 residue in conjunction with existing androgen-ablative therapy may be more effective than androgen-ablative therapy alone, in the treatment of prostate cancer patients.

Introduction

The androgens, acting via the androgen receptor (AR) play a central role in the development and maintenance of all male organs including the prostate gland (1). Their role in the emergence and progression of prostate cancer was demonstrated by seminal studies of Huggins and Hodges (2) several decades ago. Since then, endocrine therapies aiming to physically or chemically inhibit androgen signaling, by reducing circulating androgen levels and/or blocking the AR activation, constitutes the primary treatment option for prostate cancer patients (3). Though these therapies result in a temporary shrinkage of tumor mass, yet, the cancer inevitably reappears in a more aggressive, androgen-independent form known as Androgen Independent (AI) or Castration Resistant Prostate Cancer (CRPC). Despite decades of intense basic and clinical research, no treatment options are available for AI prostate cancer.

Studies reveal that despite low circulating androgen levels, the AR is activated and often expressed at higher levels in CRPC patients (4). The transition from androgen dependent to androgen-independent phenotype is attributed to a complex interplay of signaling molecules that ultimately result in aberrant androgen signaling and proliferation of tumor cells. In the absence of androgens, stimulation of tumor growth may be caused by various growth factors such as the Epidermal Growth Factor (EGF), Interleukin-6 (IL-6) or neuropeptides such as Bombesin and Gastrin releasing peptides (5). The AI stage is often associated with an increased expression of G protein-coupled receptors (GPCRs), mediators of diverse cellular responses to extracellular molecules (6). Further, the expression of GPCR ligands such as follicle stimulating hormone, endothelin 1 and neuropeptides are also higher in the prostate tumor. The higher expression of both the GPCRs and their ligands in the tumor samples indicates that probably the receptors are in the ‘on’ state in these cells and somehow contribute to the initiation or progression of the disease. Activated GPCRs mediate their signals via G proteins which belong to four groups: $G_{\alpha}$, $G_i$, $G_q$ and $G_{12}$. While $G_q$ regulates the
activity of phospholipases and intracellular Calcium levels, \( G_{12} \) regulates low molecular weight effectors such as the Rho GTPases. The \( G_s \) and \( G_i \) primarily regulate adenyl cyclases that synthesize cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA), (7). PKA consists of two regulatory (R) and two catalytic kinase subunits (C) (8). In the inactive state, the R subunits dimerize and bind to the C subunits, whereas binding of cAMP to the R subunits causes a conformational change, releasing the free and active C subunit that phosphorylates numerous cellular targets.

Studies reveal that PKA/cAMP plays a significant role in prostate cell growth and proliferation (7). PKA I is overexpressed in prostate adenocarcinoma cells and in patients with advanced prostate cancer, PKA RI overexpression was observed in 17.5% of the men and correlated significantly with poor patient outcome. Further, PKA RI overexpression predicted distal metastasis and biochemical failure in prostate cancer patients, highlighting a relationship between PKA signaling and progression to CRPC (9).

The AR is a nuclear receptor that mediates the actions of androgens such as testosterone and dihydrotestosterone. Ligand binding to the AR, leads to its dissociation from heat shock proteins in the cytosol and translocation into the nucleus where it binds to specific androgen response elements (AREs) upstream of target genes and modulates their transcription (10, 11). Several studies have demonstrated that the AR can be stimulated by high cAMP levels. AR transactivation by androgens could be enhanced by forskolin and other activators of adenylate cyclase in AR-transfected monkey kidney CV-1 cells or PC3 cells (12, 13). The increase in AR transcription was caused by enhanced AR-DNA binding, and not due to an increase in AR protein expression (1). Further, forskolin-induced PKA activation resulted in enhanced PSA expression, in the presence of a functional AR (13). The activated alpha subunit of \( G_s \) protein could also activate AR in prostate cancer cells and synergize with low concentrations of androgen to completely activate the AR through PKA (6). Though these studies indicate a definite crosstalk between the two pathways, yet, the exact role of PKA in AR activation is still unexplored.

In the present study, we demonstrate that activation of PKA is essential for nuclear translocation of AR and is a prerequisite for classical AR signaling. The unstimulated
AR is bound to HSP90 and HSP70 in the cytosol and phosphorylation of HSP90 protein at the Thr^{89} residue by PKA results in its disassociation from the AR, allowing the AR to bind to HSP27 and migrate into the nucleus.

Results

**PKA activation is necessary for AR transactivation**

Aberrant activation of AR is the primary cause of transition from androgen-dependence to androgen independence observed in patients with advanced prostate cancer (14). The cAMP/PKA pathway can induce AR in the presence of low/no androgens and remains activated in prostate cancer cells (1). In fact, androgens themselves are reported to activate PKA in prostate cells via a membrane GPCR (15). To evaluate whether activation of PKA is necessary for nuclear AR transactivation, androgen-induced ARE-regulated reporter activity was monitored in LNCaP cells in the presence and absence of PKA inhibitor H89 (30 µM, isoquinoline) or PKA-siRNA (120nM) (Fig. 1A). Pretreatment of LNCaP cells with H89, or transfection with PKA-siRNA resulted in a significant decrease in testosterone (T)-induced AR activity (Fig. 1A). Similarly, in Human Embryonic Kidney 293 cells (HEK 293), transfected with AR and ARE-luciferase, pretreatment with H89 or transfection with PKA-siRNA resulted in marked decrease in testosterone-induced reporter activity (Fig. 1B), indicating that PKA activation is critical for optimal AR transactivation. Cells over expressing scrambled siRNA did not exhibit any change in reporter activity (Fig. 1A and B).

**PKA activation is necessary for nuclear translocation of AR**

Although PKA activation is necessary for AR activity, the precise role of activated PKA in classical AR signaling needs further investigation. Studies by Kasbohm et al have indicated that PKA activation may regulate the nuclear translocation of AR (6), whereas others have indicated that PKA activation may enhance the interaction of AR with transcription factors, post-translocation (16). To investigate whether PKA activation plays a role in the nuclear translocation of AR, LNCaP cells were transfected with GFP-tagged AR, and translocation was monitored at different time points (0, 5 min, 15 min, 30 min, 1h and 4h) post testosterone treatment, in presence and absence of PKA inhibitor H89.
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(supplementary Fig.1). In the absence of testosterone, GFP-AR was mostly localized in the cytoplasm of the cells, and a time dependent import of GFP-AR into the nucleus was observed following testosterone treatment as reported earlier (17). In fact, greater than 90% of the GFP-AR had translocated completely within 1 hour of treatment (Fig.2, T and Fig. S1A). However, prior treatment of these cells with H89 (Fig. 2, T+H89 and Fig. S1B), or knocking down PKA expression using PKA-siRNA inhibited nuclear import (Fig. 2, T+siPKA and Fig. S1C), indicating the need for PKA activation in the nuclear import process of AR.

**PKA activation invokes the dissociation of AR from AR-HSP90 complex**

Molecular chaperones such as HSP90 stabilize the AR in the absence of ligand in the cytoplasm (18, 11). The AR in cytoplasm is maintained in an inactive, but responsive state, as a part of a large heterocomplex composed of heat shock proteins, co-chaperones and other proteins (1). Binding of ligand leads to a conformational change in the AR causing its dissociation from the large complex and translocation into the nucleus. Within the nucleus the AR binds to androgen response elements and recruits coactivators and other factors in the vicinity of target genes to regulate their transcription (19). Studies reveal that androgen bound AR dissociates from HSP90 but exhibits increased association with another chaperone called HSP27 (19). PKA-mediated phosphorylation could be necessary for the release of AR from hetero complex or its subsequent migration into nucleus. We monitored the relative locations of AR and HSP90 in LNCaP cells and assessed the impact of inhibition of PKA on the localization of the two molecules. LNCaP cells transfected with GFP-AR were treated with 10 nM testosterone for 1 hour and immunofluorescence studies were performed using TRITC-labeled anti HSP90 antibody. In unstimulated cells, both GFP-AR and HSP90 were localized in the cytoplasm and GFP-AR moved into the nucleus following testosterone treatment while HSP90 remained in the cytosol (Fig. 3, -T and +T). In LNCaP cells pre-treated with H89, T treatment did not induce import of GFP-AR into nucleus and both HSP90 and GFP-AR fluorescence were confined to the cytoplasm (Fig. 3, T+H89). Similarly, both HSP90 and GFP-AR colocalized predominantly in the cytosol following testosterone treatment in LNCaP cells,
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overexpressing PKA-siRNA (Fig. 3, siPKA and T + siPKA). Further, the sub-cellular location of AR and HSP90 in unstimulated (vehicle treated) and stimulated (testosterone treated) LNCaP cells and siPKA-transfected LNCaP cells was investigated by Western Blotting, post-treatment. In unstimulated LNCaP cells (vehicle treated), most of the AR and HSP90 was present in the cytoplasmic fraction, while in stimulated cells, AR was predominantly present in the nucleus while HSP90 was in the cytoplasmic fraction (Fig. 3B, LNCaP, -T, +T). Similar results were obtained in case of unstimulated and stimulated LNCaP cells transfected with scrambled siRNA (data not shown). In SiPKA-transfected LNCaP cells, AR and HSP90 were both predominantly present in the cytoplasm before and after testosterone treatment (Fig. 3B, SiPKA-LNCaP, -T, +T).

To evaluate whether PKA activation regulates the binding of AR to HSP90, co-immunoprecipitation studies were performed in LNCaP cells treated with testosterone with or without prior H89 treatment. Untreated cells were used as control. In AR immunoprecipitated complexes, probing with HSP90 antibody revealed AR and HSP90 interaction in untreated cells and dissociation of AR from HSP90 in testosterone treated cells (Fig 4A). In H89 treated cells or cells transfected with PKA-siRNA, AR-HSP90 association was intact following testosterone treatment and AR was not released from HSP90 (Fig. 4A) clearly demonstrating involvement of PKA activation step for the release of AR from HSP90. Also, HSP27-AR interaction was increased in testosterone treated cells compared to control, however, HSP27-AR association was much less in LNCaP cells treated with H89 or transfected with PKA-siRNA (Fig. 4A). Significantly, in co-immunoprecipitation experiments, immunoprecipitation with HSP90 antibody and probing with PKA substrate antibody indicated that phospho-HSP90 levels increased after testosterone treatment in LNCaP cells, whereas its levels in H89 treated or PKA-siRNA transfected cells were same as in untreated, following testosterone treatment (Fig. 4B). From the co-immunoprecipitation studies it can be concluded that AR-HSP90 interaction is severed following testosterone treatment, due to phosphorylation of HSP90 by testosterone via a PKA-mediated pathway. Also, the inhibition of PKA activation prevents HSP90 phosphorylation and consequent dissociation of AR from HSP90 complex making AR unsuitable for migration into the nucleus.
PKA activation phosphorylates the Thr$^{89}$ residue in HSP90

PKA being a kinase, could possibly regulate the AR and HSP90 complex formation by phosphorylating the AR, HSP90 or any other protein within the complex. Studies have shown that the only site in AR that is phosphorylated by PKA is Ser$^{650}$ and mutation of this Ser to Ala, S650A, did not have any significant impact on AR transactivation (6). HSP90 is phosphorylated by PKA at a single site, Thr$^{89}$ in PAEC cells (20). Mutation of this threonine residue inhibits PKA mediated phosphorylation of HSP90 and prevents the translocation of HSP90 to the surface of these cells (20). To investigate whether PKA-induced phosphorylation of Thr$^{89}$ plays a key role in the dissociation of AR-HSP90 complex, co-immunoprecipitation studies were performed in LNCaP cells transfected with HSP90 T89A (kind gift from Heitian Lei). In cells overexpressing mutant HSP90, immunoprecipitation with AR antibody, followed by immunoblotting with HSP90 antibody revealed that a significant percent of AR remained in complex with HSP90 following testosterone treatment unlike cells expressing wildtype HSP90 where complete dissociation of AR and HSP90 was observed (Fig. 4C). Since these cells also contain a small percentage of endogenous wildtype (WT) HSP90, hence, a small percentage of AR is dissociated from the HSP90 complex reflected in the minor difference in intensity of the AR-bound HSP90 product obtained in treated and untreated cells. In the same cells pre-treated with H89 or transfected with PKA siRNA, most of the AR remained in complex with HSP90 upon testosterone treatment, as in the untreated (Fig. 4C). An inverse proportionality is usually observed in the binding of HSP90 and HSP27 to AR (20). HSP27 can bind to AR only when it is released from the HSP90 complex. In keeping with this, in LNCaP cells overexpressing HSP90 T89A, only a small fraction of HSP27 was bound to AR following testosterone treatment, as most of the AR was still in complex with mutated HSP90. AR could probably uncouple only from those complexes which were formed by a few molecules of wildtype HSP90 present in these cell that could be phosphorylated. Coimmunoprecipitation studies using anti-HSP90 antibody, followed by immunoblotting with PKA substrate antibody indicated that in cells overexpressing HSP90 T89A, negligible levels of phospho-HSP90 product is obtained compared to cells that express wild
type HSP90 upon testosterone treatment. (Fig. 4D). To confirm that the observed difference in immunooblots was not due to any difference in affinity of the anti-HSP90 antibody to the wild type and mutant HSP90 proteins, Western blotting was performed using lysates from LNCaP cells transfected using HSP90, HSP90 T89A or vector alone. Results demonstrate that the antibody can detect both wild type as well as mutant HSP90 T89A efficiently (Supplementary Fig S3).

Nuclear translocation of the HSP90 T89A mutant

The migration of AR into nucleus is possible once it dissociates from HSP90 heterocomplex. In LNCaP cells overexpressing HSP90 T89A and GFP-AR, the translocation of GFP-AR was monitored by fluorescence microscope at different time intervals following testosterone treatment. While in LNCaP cells expressing wildtype HSP90 most of the AR translocated into the nucleus by 1 hour (Fig. 5, HSP90 and Fig.S2A), in cells overexpressing the HSP90 mutant, most of the AR remains in the cytoplasm, following testosterone treatment for 4 hours (Fig. 5, HSP90 T89A and Fig.S2B) or more (data not shown).

Also, in these cells, GFP-AR and mutant HSP90 colocalize in the cytoplasm before and after testosterone treatment (10 nM, 1h, Fig. 6, -T and +T). Cells pretreated with H89 or transfected with PKA-siRNA behaved in the same way as untreated and testosterone treated cells (Fig. 6, T+H89, siPKA and T+ siPKA). Collectively, these results indicate that phosphorylation of HSP90 by PKA at residue Thr^{89} is essential for the release of AR from the HSP90 complex and its subsequent migration into the nucleus.

Inhibition of AR transactivation in LNCaP cells expressing HSP90 T89A

In the cells expressing mutant HSP90, most of the AR was in complex with HSP90 and could not be imported into the nucleus. To evaluate the transcription efficiency of AR in cells overexpressing the mutant HSP90, cells were treated with increasing concentrations of testosterone and ARE regulated luciferase activity was measured and compared to that in LNCaP cells expressing wildtype HSP90. In both LNCaP and HEK293 cells, significant fall in testosterone induced reporter activity (58.56% and 56.5 % respectively) was observed in cells overexpressing HSP90 T89A as compared to those expressing wild type HSP90 (Fig. 7A and B). In fact, little
increase in reporter activity was observed following testosterone treatment in both these cell lines (Fig. 7A and B).

**Reduced cell proliferation and PSA expression in the absence of activated PKA**

Androgens play an important role in the survival and maintenance of prostate cells. Androgens also stimulate PKA activation to promote androgen signaling (15). We therefore examined whether activation of PKA is required for the proliferative effects induced by androgens in prostate cells. To confirm this, LNCaP cells were grown in testosterone deficient (control) medium or testosterone-supplemented medium, in the presence or absence of PKA-siRNA. The proliferation of cells grown in control medium was 2.9 folds in 4 days and proliferation of cells grown in testosterone was 25% more than that in control in 4 days (Fig. 8A). In cells overexpressing the PKA-siRNA, net proliferation was 2 fold in 4 days in control medium and increased by an insignificant margin following testosterone treatment (Fig. 8A). LNCaP cells transfected using scrambled siRNA proliferated in the same manner as non-transfected LNCaP cells (data not shown). In LNCaP cells overexpressing HSP90 T89A mutant, cell proliferation increased by only 2 folds in both control and testosterone treated medium compared to HSP90-transfected LNCaP cells that proliferated 2.9 folds in control medium or 3.9 folds in testosterone containing medium (Fig. 8B). We further evaluated PSA gene expression as a prototype androgen-responsive gene in LNCaP cells, under normal conditions (Fig. 8C, lanes 1 and 2), in the presence and absence of H89 (Fig. 8C, lanes 3 and 4), upon overexpression of PKA-siRNA (Fig. 8C, lanes 5 and 6) and in LNCaP cells overexpressing mutant HSP90 T89A (Fig. 8C, lanes 7 and 8). Inhibition of PKA activation in LNCaP cells by H89 or overexpressing PKA-siRNA or by overexpression of HSP90 T89A mutant caused an overall reduction in PSA expression in testosterone treated cells (Fig. 8C). In H89 treated or PKA-siRNA transfected cells, a modest increase in PSA expression could be observed upon testosterone treatment, however increase in expression was not observed in cells overexpressing HSP90 T89A mutant demonstrating the pivotal role of androgen-induced PKA activation and subsequent HSP90 phosphorylation in AR mediated gene transcription and survival of prostate cells.
AR-V7 and AR\(^{v567es}\) can induce AR signaling independent of PKA-induced HSP90 phosphorylation

Expression of wild type HSP90 is required to stabilize the AR ligand binding domain (LBD) in a high affinity androgen binding state and the loss of HSP90 function reduces much the binding affinity of AR for androgens (21). However, in clinical samples, AR variants such as AR-V7 and AR\(^{v567es}\) have been identified which lack the LBD of AR. They localize to the nucleus and cause ligand-independent transcription to induce castration-resistant growth in patients. As these variants lack LBD, they probably do not require association with HSP90 complexes for optimal hormone binding (22). However, whether HSP90 is required for initial folding of these proteins or subsequent AR transcription is not clear. We investigated the effect of overexpression of HSP90T89A mutant on the transcription of these AR splice variants. As expected, in the presence of wildtype HSP90, the ARE-regulated transcription of AR, AR-V7 and AR\(^{v567es}\) were comparable (Fig. 9A). When HSP90T89A mutant was overexpressed in these cells, the transcription of AR was reduced significantly while the transcription of AR-V7 or AR\(^{v567es}\) was not affected (Fig. 9B and C). These results clearly reveal that activation of PKA and subsequent HSP90 phosphorylation at residue Thr\(^{89}\) is critical for the transcription of wildtype AR, but not mandatory for the transcription of AR splice variants AR-V7 or AR\(^{v567es}\). Also, these results suggest that phosphorylation of HSP90 probably does not play a major role in the folding and subsequent function of these AR variants as transcription occurs unhindered in the presence of HSP90 T89A mutant.

Discussion

The importance of PKA activation for AR signaling has been demonstrated by several studies. However, the specific role of PKA and its contribution to androgen signaling remains elusive. Here we demonstrate the existence of a tripartite cross-talk between PKA, HSP90 and AR that enables dissociation of ligand-bound AR from HSP90 and its subsequent translocation into the nucleus.

We began by investigating whether the activation of PKA is required for AR-transactivation in prostate cells. ARE-regulated transcriptional activity was significantly lowered when PKA activation was inhibited, either by an inhibitor such as H89 or by over expressing PKA-siRNA,
establishing the need for normal PKA activation for AR signaling. Nuclear translocation is a pre-requisite step for the function of the AR (11). Next, we investigated whether PKA-activation was necessary for pre-translocation events or post-translocation events in the AR transcription pathway. Immunofluorescence studies performed to monitor the localization of GFP-tagged AR revealed that inhibition of PKA impeded AR nuclear import suggesting that PKA activation was an essential event prior to or during nuclear translocation.

Before ligand binding, the AR is held in cytoplasm in a heterocomplex involving HSP90 and several other proteins, that present the AR in a high affinity conformation poised for optimal ligand binding (21, 11). Ligand binding causes a conformational change in the AR that allows release and exchange of chaperones and import proteins for efficient nuclear translocation (23, 24). Studies by Zoubeidi et al suggest that subsequent to androgen treatment, the association of HSP27 with AR increases while the levels of HSP90-associated AR falls (19), suggesting that replacement of HSP90 by HSP27 is required for smooth transition of AR from the cytoplasm into the nucleus. Our co-immunoprecipitation studies support this hypothesis, and we further observe that inhibition of PKA activation either by H89 or PKA-siRNA prevents the uncoupling of AR from HSP90 and subsequent association with HSP27. The inability to dissociate from HSP90 and bind to HSP27 possibly hinders the migration of AR into the nucleus. Binding to HSP27 is required not only for AR translocation but also for interaction with AR coactivators STAT3 and ARA55 and the formation of AR transcription complex (19).

How does PKA activation cause the dissociation of AR from HSP90? PKA being a kinase is known to execute the phosphorylation of multiple cellular proteins including both the AR and HSP90 (25, 20). Since mutation of the sole PKA target sequence in AR does not have an impact on AR transcriptional activity (6), we focused on the phosphorylation of HSP90. In fact, the HSP90 T89A mutant, in which the PKA phosphorylation site is mutated, could neither support AR nuclear translocation efficiently, nor AR transactivation. Nuclear translocation of testosterone treated GFP-AR was similar to that in untreated cells. The ARE-regulated transcription levels observed in HSP90T89A overexpressing cells treated with highest concentration of
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testosterone was similar to transcription observed in unstimulated cells expressing wild type HSP90, indicating that the basal level of AR transcription decreases if PKA activation is blocked.

Our results show that androgen treatment causes phosphorylation of HSP90 in LNCaP cells, the dissociation of AR from HSP90 and its association with HSP27. Inhibition of PKA activation by H89 or PKA-siRNA reduces HSP90 phosphorylation almost to levels observed in unstimulated cells and prevents its consequent dissociation from the AR. In cells overexpressing the HSP90 T89A mutant, negligible levels of the phospho-HSP90 product is visualized as compared to cells expressing WT HSP90, as expected. As a result, much of the AR is still associated with HSP90, unlike cells expressing the WT HSP90, where complete uncoupling of AR and HSP90 is observed by immunoprecipitation studies. However, some dissociation of AR from HSP90 seems to take place in these cells, probably due to the phosphorylation of a few molecules of endogenous WT HSP90 present in them. HSP90 is phosphorylated at various residues and phosphorylation is a means of regulating HSP90 activity (26). PKA-induced phosphorylation of HSP90 at Thr89 could bring about a conformational switch in HSP90 resulting in the dissociation of AR from HSP90.

It is interesting to note that the transcriptional activity of AR variants ARV7 and ARv567es are similar in cells expressing HSP90 or HSP90T89A. These two AR variants are often identified in CRPC patients (22) and HSP90 inhibitors such as geldanamycin have little or no impact on these variants. These variants do not have the LBD of AR and hence do not need HSP90 for ligand binding. However, whether, HSP90 is required at initial stages for proper folding of these proteins or at later stages during transcription was not established (22). Since no change in ARE-regulated transcription of these splice variants was observed in cells expressing WT or mutant HSP90, it seems that phosphorylated HSP90 does not play a role in the maturation or transcriptional activity of these splice variants.

Studies in which simultaneous inhibition of PKA and androgen signaling was administered resulted in reduced prostate tumor cell proliferation as compared to AR inhibition alone (27). However, our studies indicate that instead of inhibiting the PKA pathway (using a PKA inhibitor), which may be important for the function of several proteins, the same can be achieved by
mutating a single residue in HSP90. Most of the HSP90 inhibitors developed earlier targeted the ATP-binding domain of HSP90, however, newly developed HSP90 inhibitors such as AUY922 and STA-9090, with higher specificity and less toxicity are now in clinical trials (28, 29). A small molecule inhibitor against the PKA phosphorylation site in HSP90 Thr<sup>89</sup> may be used in combination with AR inhibitors to minimize the proliferation of tumor cells in patients with advanced prostate cancer. HSP90 is a client chaperone for a variety of proteins involved in different pathways and the targeted inhibition of HSP90 along with androgen-inhibition should have restricted impact on other cellular pathways while achieving maximum impact on androgen signaling and tumor cell proliferation.

**Experimental Procedures**

**Plasmids and Reagents**

The plasmids were kindly provided as follows: human GFP-AR, PSA.61-Luc, Prof. Rakesh K Tyagi (Special Centre for Molecular Medicine, JNU, Delhi); PCMV-AR, Elizabeth Wilson (Reproductive Biology laboratory, University of North California); pLNCX2-HSP90, pLNCX2-HSP90 T89A, Hetian Lei (Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts); pLEX-HSP27, Goupei Zheng (Cancer Hospital and Institute of Guangzhou Medical University, China). Testosterone and H89 (PKA Inhibitor) were purchased from Sigma and Cayman respectively. Antibodies: AR-441, HSP90α, HSP27 were from Santa Cruz Biotechnology, PKA substrate antibody was from CST, anti-mouse and anti-rabbit HRP-conjugated secondary antibody and femtoLUCENT were from G Biosciences, Protein A agarose was from ABT.

**Cell culture**

Androgen-dependent LNCaP and COS1 cells were obtained from NCCS, Pune and were cultured in RPMI 1640 and DMEM supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (10mg/ml) respectively.

**Cell transfection and siRNA (small interfering RNA)**

For plasmid transfection, cells were cultured in 100mm petridishes up to 60-70% confluence. For single transient transfection, 1µg of plasmid was transfected with Lipofectamine 2000 transfection reagent
Cells were cultured in 100mm petridishes and transfected at 60-70% confluence using Lipofectamine 2000. Transfections were carried out with PSA.61-Luc plasmid (1µg) along with PCMV-AR plasmid (1µg). After 24 hours of transfection cells were equally split into 60mm petri dishes and allowed to attach. After 24 hours, the identical cell population was treated with testosterone (10nM) and T +H89 (30µM). Luciferase activities were measured in triplicates by using Luciferase assay reagent (Promega) and were normalized with protein concentrations of samples by the Bradford method (Hi-Media).

Fluorescence Imaging

Fluorescence imaging of live cells was performed through an Upright Olympus Optical Microscope. Cells cultured in 100mm petridishes with cover slips were transfected with GFP-AR plasmid (1µg) and treated with testosterone (10nM) and T +H89 (30µM). At different time intervals of 5 mins, 15 mins, 30 mins, 1 hour, 4 hours and 24 hours cells were visualized under microscope by mounting the coverslip on microslide.

Immunofluorescence

 INVITROGEN, 10µl per plate. Media was changed after 24 hours of transfection with complete media (with 10% FBS). Testosterone treatment was done after 24 hrs of media change.

For siRNA transfection, cells were cultured in 100mm petri dishes up to 40-50% confluence. siRNA was transfected with Lipofectamine 2000 transfection reagent. The siRNA was used to knockdown PKA. It was designed against RIα subunit of PKA (sense 5’-CCAUGGAGUCUGGCAGUACdTdT-3’, antisense 5’-GUACUGCCAGACUCCAUGGdTdT-3’). siRNA were synthesised from IDT.

MTT Assay

LNCaP cells were cultured and transfected or mock transfected with siPKA or HSP90 T89A. After 24 hours of transfection, cells (5000cells/well) were seeded in 96 well plates. Testosterone (10nM) treatment was done after 24 hours of seeding. During a time course of 24, 48, 72 and 96 hours, cell proliferation was measured using CellTiter 96 AQueous one solution reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT).

Luciferase Assay
LNCaP cells were cultured on coverslips and transfected with GFP-AR (5µg) along with or without siPKA (120nM)/ pLNCX2-HSP90 T89A (2µg). Media was changed after 24 hours of transfection with complete media (with 10% FBS). Cells were treated with T/T+ H89 for 1 hour. After treatment, cells were fixed with ice-cold 100% methanol for 10 mins pre-cooled at -20 °C. Cells were then washed thrice with PBS and incubated with 0.2% Triton/PBS for 10 mins, followed by PBS washing and 30 min blocking in 3% BSA/PBST. After blocking, cells were incubated overnight in antibody HSP90 (1:100) at 4°C. Cells were visualized using anti-mouse antibody coupled to rhodamine (1:500, 1 hour). Images were captured with upright Olympus Optical Microscope with 100X objective.

**Reverse Transcription PCR**

Cells were cultured and transfected with siPKA/ HSP90 T89A and after 24 hours media was changed with complete media. After 24 hours of transfection, cells were treated/non-treated with T/T+H89. The total cell RNA was isolated using Trizol based isolation method. Cells were lysed in trizol reagent (1ml). To this lysate, 200µl of chloroform was added, shaken vigorously and incubated at room temperature (RT) for 5 mins. The solution was centrifuged at 12,000 rpm for 15 mins at 4°C. The top aqueous layer was collected carefully and precipitated by adding 500µl of isopropanol followed by incubation for 10 mins at RT. The precipitated RNA was collected by centrifugation at 12,000 rpm for 10 mins at 4 °C. The ethanol was drained off and pellet was air-dried resuspended in 50µl of RNase free DEPC water. 1µg of RNA obtained was used for cDNA synthesis using Biorad Iscript cDNA synthesis kit and used for quantitative RT-PCR analysis for PSA expression.

**Co-Immunoprecipitation**

Cells were harvested for immunoprecipitation. Whole cell lysate was prepared using cell lysis buffer containing 20 mM Tris/HCl (ph 7.5), 150 mM NaCl, 1% Nonidet P40, 1mM Dithiothreitol with protease inhibitors Aprotinin (10µg), Phenylmethane Sulfonyl fluoride (10µg) and phosphatase inhibitor cocktail (10µl), then lysate were centrifuged at 14,000 rpm at 4°C for 15 mins. The pellet was discarded and supernatant was used as whole cell lysate for immunoprecipitation. The protein concentrations were measured using Bradford kit (Himedia). The proteins in cell lysate were immunoprecipitated with 2µg of
AR antibody (Santa Cruz) for overnight at 4°C. The immune complexes were recovered with Protein A agarose for 1.5 hours with rotation. The resin was pelleted, washed with PBS (phosphate buffer saline) thrice and submitted to SDS/PAGE, followed by western blotting.

**Immuoblotting**

The samples separated by SDS/PAGE were transferred to PVDF membrane (MDI). The membrane was blocked in TBST (Tris buffered saline/Tween 20, 20mM Tris/HCl ph-7.5, 150mM NaCl, 0.1% Tween 20) with 5% Non-fat dry milk for 1 hour at room temperature (RT). The membrane was incubated with Primary antibody diluted in TBST with 3% non-fat dry milk for overnight at 4°C. Then washed membrane three times with TBST for 15 mins each and incubated with HRP-conjugated secondary antibody for 1.5 hours with rotation at RT. Following three washes with TBST, immunoreactive bands were detected by femtoLUCENT (G Biosciences).

**Statistical Analysis**

Experiments were repeated at least three times. Data are expressed as mean ± S.E. Statistical analysis was performed using Student’s ‘t’ test and graphs were generated using Prism (version 5.0).

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Figure Legends

Figure 1. Inhibition of PKA activation inhibits the testosterone regulated transactivation of AR.  A. LNCaP cells were co-transfected with PSA.61-Luc with or without siPKA (120nM)/scrambled siRNA (100nM).  B. HEK293 cells were co-transfected with PCMV-AR and PSA.61-Luc plasmids with or without siPKA(120nM). Transfected cells were pretreated with H89 (30µM) for 40 mins prior to treatment with increasing concentrations of testosterone (10pM,
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0.1nM, 1nM, 10nM, 100nM, 1000nM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from three independent experiments performed in duplicate.

**Figure 2. Inhibition of AR translocation using PKA Inhibitor, H89 and by PKA-specific siRNA (SiPKA) in LNCaP cells.** LNCaP cells were transfected with GFP-AR with or without siPKA (120nM). Transfected cells were treated or not treated with H89 (30μM) for 40 mins prior to treatment with testosterone (10nM). A. Images at 0 min and 60 mins time interval after treatment with Testosterone. B. Images after treatment with testosterone in the presence of H89. C. Images of siPKA transfected cells after treatment with Testosterone. Scale bar, 10μm (added using Image J).

**Figure 3. Colocalization of HSP90 and AR in presence and absence of H89 and siPKA:** LNCaP cells were transfected with GFP-AR (5μg) with or without siPKA (120nM). Transfected cells were treated using vehicle or testosterone (10nM) for 1 hour. H89 (30μM) treatment was done 40 mins prior to testosterone treatment. HSP90 localization was determined by Immunofluorescence staining. Nuclei were stained using Hoechst. Scale bar, 10μm (added using Image J). B. Cytoplasmic and nuclear fractionation of AR and HSP90 in unstimulated (vehicle treated) and testosterone-stimulated cells in presence and absence of siPKA. LNCap cells transfected with AR and HSP90 with or without siPKA (120nM) were treated using vehicle or testosterone (10nM) for 2 hours. Western blot was done with 50μg of protein using AR or HSP90 antibody. GAPDH was used as loading control.

**Figure 4. Androgens lead to HSP90 phosphorylation via the PKA signaling pathway:** A. Inhibition of PKA activation increases HSP90 levels in complex with AR as HSP27 decreases. LNCaP cells were co-transfected with PCMV-AR, pLNCX2-HSP90, pLEX-HSP27 with or without siPKA (120nM). Cells were treated using Testosterone (10nM) for 1 hour, post-transfection. 300μg of total extract were immunoprecipitated using AR antibody, and western blot was done using HSP90, HSP27 and AR antibodies. B. Testosterone promotes PKA dependent phosphorylation of HSP90. LNCaP cells transfected with or without siPKA (120nM) were treated or not treated with testosterone (10nM) for 1 hour. 250μg of total extract were immunoprecipitated with HSP90 antibody and western blot was done using PKA substrate and HSP90 antibody. C. Mutated HSP90 T89A increases HSP90 levels in complex with AR as HSP27 decreases. LNCaP cells were co-transfected with PCMV-AR, pLNCX2-HSP90 T89A,
pLEX-HSP27 with or without siPKA (120nM). After transfection, cells were treated using 10 nM Testosterone for 1 hour. 300μg of total extract were immunoprecipitated using AR antibody, and western blot was performed using HSP90, HSP27 and AR antibodies. D. PKA phosphorylation does not take place in presence of HSP90 T89A. LNCaP cells transfected with or without pLNCX2-HSP90 T89A were treated or not treated with testosterone (10nM) for 1 hour. 250μg of total extract were immunoprecipitated with HSP90 antibody and western blot was done using PKA substrate and HSP90 antibody.

**Figure 5. Inhibition of AR translocation in HSP90 T89A transfected LNCaP cells.** LNCaP cells were transfected using GFP-AR alone or in combination with HSP90 T89A mutant. Transfected cells were treated with testosterone (10nM) and images were captured. A. Images at 0 min and 60 mins time interval after treatment with Testosterone (10nM). B. Images captured at 0 min and 60 mins time interval post Testosterone treatment in HSP90 T89A transfected cells. Scale bar, 10μm (added using Image J).

**Figure 6. Colocalization of HSP90 T89A and AR in presence and absence of H89 and siPKA:** LNCaP cells were transfected with GFP-AR (5μg) and HSP90 T89A with or without siPKA. Media was changed after 24 hours of transfection. Transfected cells were treated with testosterone (10nM) for 1 hour. HSP90 localization was determined using immunofluorescence staining. Nuclei was stained with Hoechst. Scale bar, 10μm (added using Image J).

**Figure 7. Inhibition of AR transcription in the presence of HSP90 mutant:** A. LNCaP cells were transfected with PSA.61-Luc and pLNCX2-HSP90/ pLNCX2-HSP90T89A. B. HEK293 cells were transfected with PCMV-AR, PSA.61-Luc and pLNCX2-HSP90/ pLNCX2-HSP90T89A. Transfected cells were treated with increasing concentrations of testosterone (10pM, 0.1nM, 1nM, 10nM, 100nM, 1000nM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from three independent experiments performed in duplicate.

**Figure 8. A. Androgen-induced LNCaP cell proliferation and PSA expression requires PKA activation.** LNCaP cells that were transfected or not transfected with siPKA (120nM), were treated with testosterone (10nM) for 24, 48, 72 and 96 hours and cell growth rates were determined by MTT assay. B. Transfection of HSP90 T89A mutant inhibits LNCaP cell proliferation. HSP90 T89A transfected and mock transfected cells were treated with testosterone
(10nM) for 24, 48, 72 and 96 hours and cell growth rates were determined by MTT assay. C. Testosterone-induced PSA expression in LNCaP cells requires PKA activation: LNCaP cells transfected or not transfected with siPKA/HSP90 T89A were treated with testosterone (10nM) or vehicle alone for 24 hours. Total RNA was isolated and expression of PSA, an AR target gene was measured by RT-PCR, β-actin was used as control.

**Figure 9. A. Increase in transcription of truncated AR variants (ARV7 and ARv567es) following Testosterone treatment in cells expressing wild type HSP90 or HSP90T89A.** HEK293 Cells transfected with PSA.61-Luc and PCMV-AR/pCDNA-ARV7/pCDNA-ARv567es plasmids were treated with increasing concentrations of testosterone (10pM, 0.1nM, 1nM, 10nM, 100nM, 1000nM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from three independent experiments performed in duplicate. **B & C. Mutated HSP90 does not affect the transcription of truncated AR variants (ARV7 and ARv567es).** HEK293 cells transfected with PSA.61-Luc, PCMV-AR/pCDNA-ARV7/pCDNA-ARv567es and HSP90/HSP90T89A plasmids were treated with increasing concentrations of testosterone (10pM, 0.1nM, 1nM, 10nM, 100nM, 1000nM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from three independent experiments performed in duplicate.
Figure 1

A

- Testosterone
- Testosterone + H89 30μM
- Scrambled siRNA
- SiPKA

B

- Testosterone
- Testosterone + H89
- T + siPKA
Figure 2
Figure 3
Figure 4
Figure 5

Figure 6
Figure 7
Figure 8
Figure. 9
Phosphorylation of HSP90 by protein kinase A is essential for the nuclear translocation of androgen receptor
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