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# Inhibition of Glycogen Synthase Kinase-3β Promotes Nuclear Export of the Androgen Receptor Through a CRM1-Dependent Mechanism in Prostate Cancer Cell Lines

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## ABSTRACT

The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the steroid hormone receptor superfamily. Under normal conditions, in the absence of a ligand, the AR is localized to the cytoplasm and is actively transported into the nucleus upon binding of androgens. In advanced prostate cancer (PCa) cell lines, an increased sensitivity to dihydrotestosterone (DHT), enabling the cells to proliferate under sub-physiological levels of androgens, has been associated with increased stability and nuclear localization of the AR. There is experimental evidence that the glycogen synthase kinase-3β (GSK-3β), a multifunctional serine/threonine kinase is involved in estrogen and AR stability. As demonstrated in the following study by immunoprecipitation analysis, GSK-3β binds to the AR forming complexes in the cytoplasm and in the nucleus. Furthermore, inhibition of GSK-3β activity by pharmacological inhibitors like the maleimide SB216761, the chloromethyl-thienyl-ketone GSK-3 inhibitor VI or the aminopyrazol GSK-3 inhibitor XIII in cells grown in the presence of DHT triggered a rapid nuclear export of endogenous AR as well as of green fluorescent AR-EosFP. The nuclear export of AR following GSK-3β inhibition could be blocked by leptomycin B suggesting a CRM1-dependent export mechanism. This assumption is supported by the localization of a putative CRM1 binding site at the C-terminus of the AR protein. The results suggest that GSK-3β is an important element not only in AR stability but also significantly alters nuclear translocation of the AR, thereby modulating the androgenic response of human PCa cells. J. Cell. Biochem. 109: 1192–1200, 2010. 

**KEY WORDS:** GSK-3B; ANDROGEN RECEPTOR; NUCLEAR EXPORT; CRM1

Prostate cancer (PCa) is the most commonly diagnosed neoplasm in elderly man and a major cause of cancer-related deaths in the Western world. In their early stages, most PCas respond to androgen deprivation therapy achieved by surgical or medical castration reflecting the dependence of PCa cells on androgenic stimuli for growth and survival. Although this therapy is initially very effective, almost all tumors relapse to a hormone refractory stage where tumor cells can grow under castrate levels of androgens. The underlying mechanisms for hormone refractory prostate cancer (HRPCa) are poorly understood. In the past it was presumed that the expression of the androgen receptor (AR) is lost in the cells of advanced, hormone-refractory tumors. However, clinical studies demonstrated that the AR is rarely lost in human PCa specimens in vivo, even in those of therapy-refractory tumors [Hobisch et al., 1995]. Both, gene of function mutations as well as AR amplification have been reported in HRPCa. Moreover, it had also been proposed that peptide growth factor or cytokine signaling pathways could activate or enhance AR signaling, especially in the presence of low levels of circulating androgens [Cronauer et al., 2003]. In advanced PCa cell lines, an increased sensitivity to dihydrotestosterone (DHT), enabling cells to proliferate under sub-physiological levels of androgens, has been associated with increased stability and/or nuclear localization of the AR [Umekita et al., 1996; de Vere White et al., 1997; Gregory et al., 1998, 2001; Sweat et al., 1999]. In summary, current pre-clinical and clinical studies imply that the AR is not only expressed but is still transcriptionally active in the majority of recurrent PCa after failure of hormone deprivation therapy [Scher et al., 2004; Snoek et al., 2009].

The AR is a member of the nuclear receptor superfamily and acts as a ligand-dependent transcription factor. The AR protein consists of 919 amino acids and has a molecular weight of around 120 kDa. Structurally, the receptor is organized in four different domains: the

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N-terminal transactivation domain (NTD), the central DNA-binding domain (DBD) containing two zinc-finger motifs, the C-terminal ligand-binding domain (LBD) and the hinge-region which connects DBD and LBD [Cronauer et al., 2003]. In the absence of androgens, the AR is located in the cytoplasm associated with heat shock proteins (HSPs). Upon stimulation with androgens, HSPs dissociate, the AR is phosphorylated, dimerizes, and translocates to the nucleus, where it binds to androgen-responsive elements (AREs) on the DNA and thus regulates transcription of androgen-dependent genes [Feldman and Feldman, 2001].

One potential mechanism by which AR stability and function may be increased is by post-translational modification like phosphorylation. Indeed the AR has been shown to express potential phosphorylation sites at serine, threonine, and tyrosine residues [Gioeli et al., 2002; Wong et al., 2004; Guo et al., 2006]. Alteration of AR phosphorylation by protein kinases are thought to provide a mechanism enabling the cells to circumvent the inhibitory effects caused by androgen ablation therapy [McCall et al., 2008]. There is experimental evidence that the glycogen synthase kinase-3 (GSK-3) is involved in AR signaling [Liao et al., 2004; Mazor et al., 2004; Rinnab et al., 2008]. The GSK-3 is a ubiquitously expressed serine/threonine kinase with orthologs identified in nearly every eukaryontic species. In humans, two highly homologous forms of the enzyme, GSK-3α and GSK-3β have been isolated. These two isoforms were reported to exhibit about 97% sequence homology within their kinase domain [Woodgett, 1990]. Initially identified by its ability to phosphorylate and inactivate glycogen synthase, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate a broad range of proteins, including members of the steroid receptor superfamily [Rogatsky et al., 1998; Salas et al., 2004; Medunjanin et al., 2005]. As recently shown in vitro, GSK-3β is involved in the regulation of estrogen and AR stability and function [Mazor et al., 2004; Medunjanin et al., 2005; Grisouard et al., 2007; Rinnab et al., 2008].

The following study demonstrates that GSK-3β binds to the AR forming a complex in the cytoplasm that seems to be transported into the nucleus upon androgenic stimulation. Inhibition of GSK-3β activity by structurally and functionally different pharmacological inhibitors triggered a rapid nuclear export of the AR in androgen-stimulated PCa cells. In contrast to the relatively slow export of the AR from the nucleus after androgen withdrawal (>8 h) nuclear export triggered by GSK-3 inhibitors was already detectable after 30 min reaching its maximum after 4 h. The induction of the fast AR nuclear export was independent of the mechanism of action of the GSK-3 inhibitor used and could be blocked by leptomycin B (LMB), the latter suggesting a CRM1-dependent export mechanism. This assumption was furthermore supported by the localization of a CRM1 binding site at the carboxyterminal end of the AR. The results suggest that inhibition of GSK-3β helps target the AR for export from the nucleus and thereby modulates the androgenic response of human PCa cells.

## MATERIALS AND METHODS

### CHEMICALS

GSK-3β inhibitor SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione) was provided by Biomol GmbH (Hamburg, Germany). GSK-3β inhibitors VI (2-chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone) and XIII (5-methyl-1H-pyrazol-3-yl-2-phenylquinazolin-4-yl-amine) as well as phosphatase inhibitor cocktail set III were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). Protease inhibitors Pevabloc, Aprotenin, Leupeptin, and Pepstatin were purchased from AppliChem GmbH (Darmstadt, Germany). LMB was a product of Alexis Biochemicals (Lörrach, Germany). DHT, ethylene-diamine-tetraacetic acid (EDTA), hydroxyethyl-piperazineethanesulfonic acid (HEPES), dithiotreitol (DTT), and Nonidet-P40 (NP-40) were provided by Sigma–Aldrich GmbH (Taufkirchen, Germany). Formalin and glycerol were products of Roth GmbH (Karlsruhe, Germany). Bovine serum albumin (BSA) was provided by PAA Laboratories GmbH (Pasching, Austria). All other chemicals, if not specified, were purchased from Sigma–Aldrich GmbH.

### PLASMIDS

pGL3Eprob, a probasin promoter luciferase reporter plasmid, including a 267-bp fragment of the rat probasin gene promoter (base positions −256 to +11), was kindly provided by Dr. Zoran Culig (Innsbruck, Austria). *Renilla reniformis* luciferase reporter Plasmid (pRL-tk-LUC), used as an internal control for transfection efficiencies, was purchased from Promega (Mannheim, Germany). Plasmid pAR-t1EosFP, an expression vector coding for the green fluorescent AR-fusion protein AREos, was a gift from Dr. Jörg Wiedenmann (Ulm University, Germany).

### ANTIBODIES

Mouse monoclonal AR antibody (AR 441), directed against the N-terminal domain of the AR, was purchased from Dako GmbH (Hamburg, Germany). Rabbit monoclonal antibody (Clone EP670Y) directed against the C-terminus of the AR was a product of Epitomics (Biomol). C-terminally directed AR antibody (AR C19, polyclonal rabbit antibody), rabbit polyclonal Lamin A antibody (H-102), and rabbit polyclonal antibody (H300) to CRM1 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-GSK-3β (27C10) was a product of Cell Signaling Technology (Frankfurt a. M., Germany). Monoclonal mouse antibodies to phosphoserine (clone 1C8) and phosphothreonine (clone 1E11) were products of NanoTools Antikoerpertechnik GmbH (Teningen, Germany). Mouse monoclonal antibody AC-15 to β-actin and goat anti-mouse IgG peroxidase conjugate were provided by Sigma–Aldrich GmbH. Horseradish peroxidase-labeled goat anti-rabbit antibody was a product of Pierce (Rockford, IL).

### CELL CULTURE

AR-positive LNCaP and 22Rv1 PCa cell lines, and AR-negative PC3 cells were provided by the American Type Culture Collection (Manassas, VA). RPMI-1640, phosphate-buffered saline (PBS), and penicillin/streptomycin solution were products of PAA Laboratories GmbH. Fetal bovine serum (FBS) and steroid-free dextran-charcoal-treated FBS (FBSdcc) were obtained from BioWest (Nuaille, France). Cell culture plastic ware was purchased from Sarstedt (Nürmbrecht, Germany) or Nunc (Langenselbold, Germany).

22Rv1, LNCaP, and PC3 PCa cells were routinely cultured in RPMI-1640, supplemented with 1% penicillin/streptomycin (v/v), and 10% FBS (v/v). During experiments, cells were maintained in

RPMI-1640 with 2.5% FBSdcc (v/v) and antibiotics in the presence/absence of DHT and the respective inhibitors.

### REPORTER GENE ASSAYS

Androgen receptor signaling was analyzed by an AR-specific reporter gene assay as recently described [Rinnab et al., 2008]. In brief, AR-positive 22Rv1 cells were transiently co-transfected with pGL3Eprob and pRL-tk-LUC using FuGene HD (Roche Diagnostics Corporation, Basel, Switzerland). Twenty-four hours after transfection, cells were treated with different GSK-3 inhibitors in the presence/absence of 10 nM DHT. Reporter gene activity was assessed after a 24-h incubation period using the Dual-Luciferase Reporter Assay [Rinnab et al., 2008].

### NUCLEAR TRANSLOCATION ASSAYS

Nuclear translocation of AR was analyzed in PC3 cells transfected with pAR-t1EosFP coding for a green fluorescent AR-Eos-fusion protein (AR-EosFP) [Cronauer et al., 2007]. Twenty-four hours after transfection, cells were treated with/without leptomycin, DHT and GSK-3 inhibitors, SB216763, GSK-inhibitor VI or GSK-inhibitor XIII for 4 h. Subsequently, fluorescent cells were counted and AR distribution was categorized as nuclear staining, cytoplasmic staining or staining in both compartments. For reasons of clarity only the percentage of nuclear staining ± standard deviation (SD) is shown in the corresponding graphical figure.

### PREPARATION OF NUCLEAR AND CYTOSOLIC EXTRACTS

Monolayers from cells grown in T25 flasks were lysed in 500 µl buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP-40, pH 7.9) supplemented with phosphatase and protease inhibitors. Subsequently cells were scraped thoroughly out of the flask and left on ice for 10 min. Cell lysates were centrifuged at 3,000*g* for 10 min at 4°C in a tabletop centrifuge. Supernatants containing the cytosolic fraction were collected and transferred into a separate tube. Residual pellets containing the nuclei were resuspended in 93.5 µl buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9) and 6.5 µl of 4.6 M NaCl and vortexed for 30 s at highest settings. After incubation on ice for another 30 min, nuclear lysates were centrifuged at 15,000*g* for 30 min at 4°C to remove nuclear debris. Protein concentrations of cytosolic and nuclear extracts were determined using the BCA-Protein Assay (Pierce).

### PREPARATION OF IMMUNOPRECIPITATES

GSK-3β/AR complexes of nuclear/cytoplasmatic fractions (containing 100 µg protein) were immunoprecipitated with 1 µg rabbit anti-GSK-3β (27C10) while gently rotating at 4°C for 1 h on a roller mixer (Stuart ST6, VWR International, Darmstadt, Germany). After that, 20 µl of Protein A/G-Sepharose (Santa Cruz Biotechnology) were added and the mixture was incubated overnight at 4°C while rotating. Subsequently, sepharose beads were washed three times with PBS and resuspended in 40 µl sample buffer (100 mM Tris–HCl, 3% SDS, 10% glycerol, 2% β-mercaptoethanol, 1 mg/ml bromphenol blue). Twenty microliters of this solution were subjected to Western blot analysis.

CRM1/AR complexes: Whole cell extracts of LNCaP and 22Rv1 cells (containing 100 µg of protein) were immunoprecipitated with 1 µg rabbit polyclonal CRM1-antibody (H300) according to the procedure described above.

Serine/threonine phosphorylation of the AR was analyzed in whole cell extracts of 22Rv1 cells. Therefore the cells were grown for 30 min in the presence/absence of 1 µM SB216763 followed by a 60 min incubation with/without 10 nM DHT. Whole cell extracts (containing 250 µg protein) were immunoprecipitated with 1 µg AR-C19 antibody, directed against the C-terminal end of the AR, as described above.

### WESTERN BLOT ANALYSIS AND IMMUNODETECTION

Cell extracts or immunoprecipitates were electrophoresed in a 10% SDS–PAGE and electroblotted onto nitrocellulose membranes as recently described [Rinnab et al., 2008]. AR protein was detected using AR-specific antibodies recognizing either the N- or C-terminal epitopes of the AR (N-terminal: AR441 monoclonal mouse, 1:1,000 and C-terminal: AR rabbit monoclonal antibody, Clone EP670Y, 1:2,000). AR phosphorylation was analyzed in AR immunoprecipitates using monoclonal mouse antibody to phosphoserine (clone 1C8, 1:100) or monoclonal mouse antibody to phosphothreonine (clone 1E11, 1:100). Incubation with rabbit Lamin A antibody (H-102, 1:1,000) or mouse monoclonal antibody AC-15 to β-actin (1:20,000) served as a loading control for nuclear or cytoplasmatic fractions, respectively. Immunoreactive bands were visualized using horseradish peroxidase-labeled goat anti-rabbit antibody (1:2,000) or goat anti-mouse antibody (1:5,000) as recently described [Rinnab et al., 2008].

### STATISTICAL ANALYSIS

All experiments were carried out at least three times. Data are reported as mean ± SD. Analysis was performed with Student's *t*-test with $P < 0.05$ considered as significant.

## RESULTS

### INTERACTION OF GSK-3β AND AR IN PROSTATE CANCER CELLS

GSK-3β has been found in the cytoplasm as well as in the nucleus of different cell lines [Salas et al., 2004; Medunjanin et al., 2005; Grisouard et al., 2007]. Moreover, in whole cell lysates from AR- and GSK-3-over-expressing cells, GSK-3β was shown to bind to the AR [Mazor et al., 2004]. In order to analyze whether GSK-3 binding to the AR depends on androgenic stimuli, cytoplasmic and nuclear fractions of AR-positive human PCa-cell lines LNCaP and 22Rv1 were subjected to co-immunoprecipitation (IP) analysis in the presence/absence of androgens. As shown in Figure 1, in the absence of androgens, co-immunoprecipitates of GSK-3β/AR were predominantly found in the cytoplasm although complex formation was also observed in the nucleus. In contrast, when adding DHT to the cells, the strongest GSK–AR signals could be detected in the nuclei of both cell lines whereas cytoplasmic co-immunoprecipitates were decreasing under these conditions (Fig. 1). In summary, these findings strongly suggest that GSK-3 and AR form complexes both in the cytoplasm as well as in the nucleus and that a possible co-transport of both proteins could not be excluded.

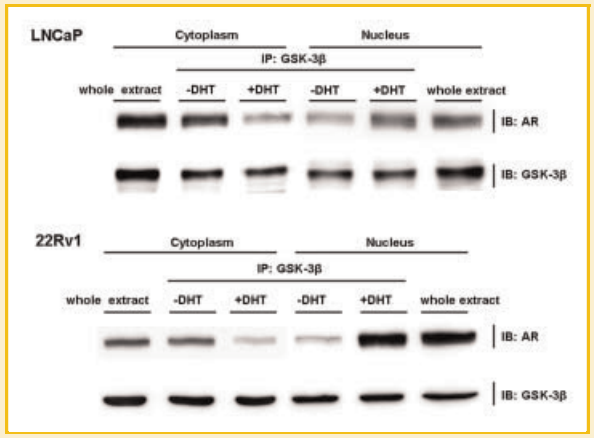


Fig. 1. GSK-3β binds to the AR. Analysis of AR–GSK-3 interaction by IP of nuclear and cytosolic cell fractions: LNCaP and 22Rv1 cells were grown in T25 flasks for 24 h in RPMI-1640 supplemented with 10% FBS (v/v) and antibiotics. After that, the medium was replaced by RPMI-1640, 10% FBSdcc (v/v), and antibiotics for another 24 h. Subsequently, cells were grown in the presence/absence of 10 nM DHT for 4 h. One hundred micrograms of nuclear/cytosolic protein extracts was subjected to immunoprecipitation (IP) using an antibody directed against GSK-3β followed by Western blot analysis with anti-AR and anti-GSK-3β antibodies (IB) as described in the Materials and Methods Section. IP: GSK-3 (anti-GSK-3β, 27C10), IB: AR (anti-AR, AR441) or GSK-3β (anti-GSK-3β, 27C10 serving as loading control for IP).

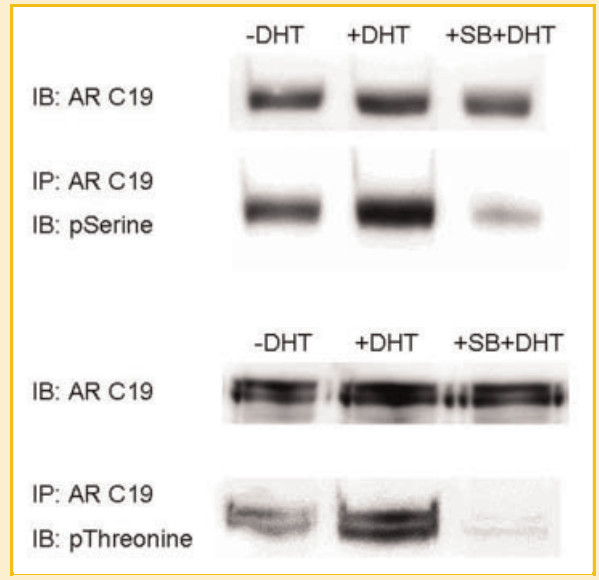


Fig. 2. SB216763 prevents serine and threonine phosphorylation of the AR. 22Rv1 cells were cultured in T25 flasks for 24 h under standard conditions (RPMI-1640 supplemented with 10% FBS (v/v) and antibiotics). Thereafter medium was replaced and cells were grown for 24 h under steroid free conditions (RPMI-1640, 10% FBSdcc, antibiotics). Subsequently, cells were pre-incubated with/without 1 μM SB216763 30 min before addition of 10 nM DHT. Proteins were collected 1 h after addition of DHT. Two hundred fifty micrograms of total protein extract was immunoprecipitated with 1 μg AR-C19 antibody. Immunoprecipitates were analyzed by monoclonal antibodies recognizing phosphoserine or phosphothreonine residues.

## INHIBITION OF GSK-3 BY SB216763 PREVENTS SERINE/THREONINE PHOSPHORYLATION OF THE AR IN 22RV1 CELLS

The finding that GSK-3 interacts with the AR in the cytoplasm as well as in the nucleus prompted us to analyze the effects of GSK-3 on AR phosphorylation. As can be seen in Figure 2 treatment of the 22Rv1 cells with DHT induces AR phosphorylation on serine- and threonine sites. Pre-treatment of the cells with the GSK-3 inhibitor SB216763 leads to a dramatical inhibition of both serine and threonine phosphorylation of the AR in vivo, indicating that GSK-3 plays an important role in AR phosphorylation.

## INHIBITION OF GSK-3 TRIGGERS A RAPID NUCLEAR EXPORT OF FULL LENGTH AR PROTEIN VIA A CRM1-DEPENDENT MECHANISM

As previously shown by our group, GSK-3 inhibitors SB216763 and XIII were able to induce a rapid nuclear export (>30 min) of transiently over-expressed AR-EosFP in DHT-treated PC3 cells [Rinnab et al., 2008]. The underlying mechanism for this phenomenon remained largely unknown. In order to determine, whether the nuclear export of the AR by GSK-3 inhibitors depends on the most prominent export receptor, the chromosome region maintenance 1 (CRM1), nuclear translocation experiments were repeated in the presence/absence of LMB (Fig. 3A). LMB prevents the interaction between a specific nuclear export signal (NES) of a protein and the CRM1 receptor and, therefore, inhibits nuclear export of target proteins. In PC3 cells transfected with AR-EosFP, the receptor protein was predominantly located in the cytoplasm in the absence of androgens. Upon androgenic stimulation, AR-EosFP was rapidly transported to the nucleus (Fig. 3B) reaching 94% nuclear staining after 30 min of treatment with DHT. The latter could be almost completely reversed by the addition of SB216763 (7% nuclear staining after a 240 min SB216763 treatment) (Fig. 3B). Most interestingly SB216763 triggered export of the AR could be prevented by pre-treatment with LMB (92% nuclear staining) (Fig. 3B), indicating a CRM1 dependent export mechanism. In order to ascertain that the rapid export of the AR is not due to unexpected side effects of SB216763 we inhibited the GSK-3 by the aminopyrazol GSK-3 inhibitor XIII. As seen in Figure 3B nuclear export of AR-EosFP could also be achieved when using GSK-3 inhibitor XIII instead of SB216763 (Fig. 3B).

SB216763 and GSK-3 inhibitor XIII operate in an ATP-competitive manner [Meijer et al., 2004]. In consequence we analyzed the effects of a specific, non-ATP competitive GSK-3β inhibitor, the chloromethyl-thienyl-ketone (GSK-3 inhibitor VI) on the nuclear export of the AR. Like SB216763 and XIII, the GSK-3 inhibitor VI was able to induce a rapid export of AR-EosFP from the nucleus (Fig. 3B). Moreover, the effects of GSK-3 inhibitor VI could be reversed by pre-incubation with LMB (Fig. 3B). The observation that structurally and mechanistically different GSK-3 inhibitors are able to induce an LMB reversible nuclear export of Eos-AR strongly supports the assumption that inhibition of GSK-3 activity is responsible for the nuclear export of AR by a CRM1 dependent mechanism (Fig. 3B). It is interesting to note that, in contrast to GSK-3β-inhibition, androgen withdrawal caused only a slow and LMB insensitive nuclear export of the AR suggesting two distinct export mechanisms for the AR in PCa cells (see Supplemental Data).

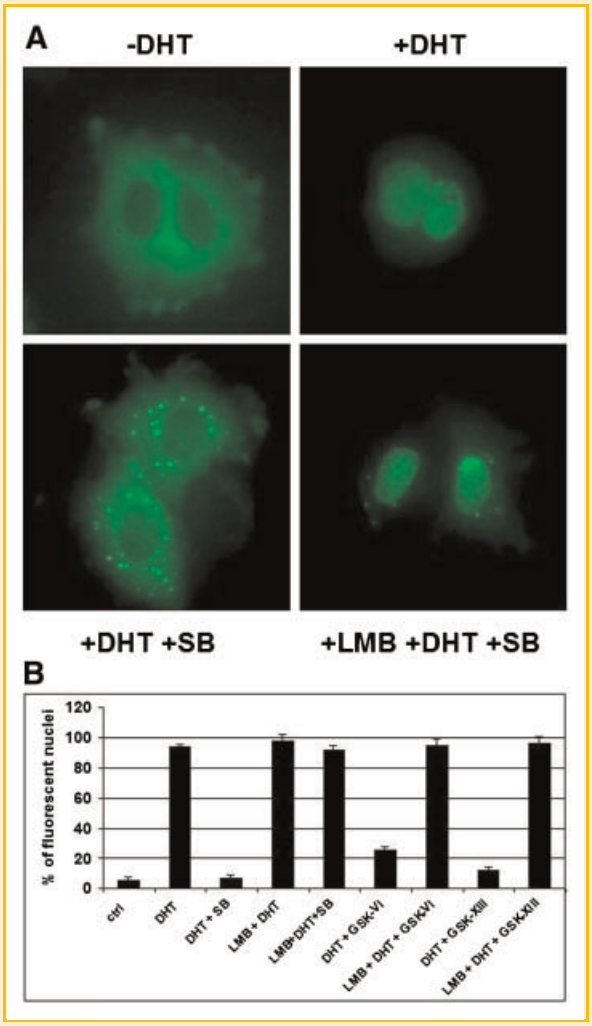


Fig. 3. Nuclear export of AR due to GSK-3 inhibition is mediated by a CRM1-dependent mechanism. AR-negative PC3 cells were seeded in 24-well plates and allowed to adhere overnight. Cells were transfected with pAR-t1EosFP and allowed to grow for another 24 h under steroid free conditions. Thereafter, cells were treated with/without 1 ng/μl LMB 30 min prior to the addition of 10 nM DHT. Thirty minutes later, GSK-3 inhibitors, SB216763, VI, or XIII, were added at 1 or 10 μM (GSK-VI and XIII). AR-EosFP localization was measured 4 h later by fluorescence microscopy. (A) Localization of AR Eos-FP. (B) Fluorescent cells were counted by fluorescent microscopy (30 cells/well). Results are expressed as a percent of green fluorescent nuclei ± SD.

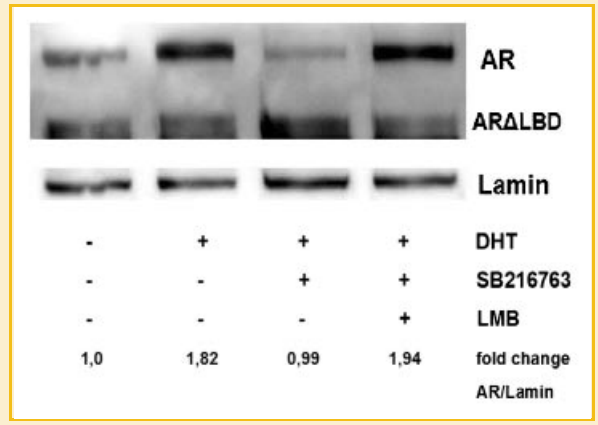


Fig. 4. Nuclear export of endogenous AR following GSK-3 inhibition is mediated by a CRM1 dependent mechanism. 22Rv1 cells were seeded in T25-flasks and allowed to grow overnight. Twenty-four hours after seeding, medium was changed to FBSdcc. Forty-eight hours after seeding, cells were pre-treated with 1 ng/μl LMB if necessary for 30 min, before addition of 10 nM DHT. After 30 min, SB216763 was added at 1 μM. Four hours later, cytoplasmic and nuclear fractions were collected as described in the Materials and Methods Section. Nuclear AR (full length AR and ARΔDBD) was detected by Western blotting with the AR441 antibody and Lamin A as a loading control. AR and Lamin A-signals were quantified and expressed as fold-change AR/Lamin.

The finding that different GSK-3 inhibitors are able to enhance a rapid nuclear export of transiently over-expressed AR-EosFP prompted us to ascertain that this effect is also true for endogenously expressed AR. Therefore, nuclei prepared from AR-positive 22Rv1 were studied by Western blot analysis (Fig. 4). 22Rv1 cells express two AR variants, a mutated full length AR with duplicated exon 3 (ARex3dup: 122 kDa) and a shortened 79 kDa AR variant termed ARΔLBD lacking the LBD in the C-terminal part of the AR [Tepper et al., 2002; Cronauer et al., 2004]. Whereas ARΔLBD exhibits constitutive nuclear localization and DNA binding, these functions remain androgen dependent in the full length AR [Tepper et al., 2002; Hartel et al., 2004].

Treatment with DHT dramatically increased nuclear full length AR in cells (AR/Lamin = 1.82) as compared to cells grown in the absence of DHT (AR/Lamin = 1.00). When adding SB216763 to DHT-treated cells, endogenous ARex3dup was rapidly exported from the nucleus reaching the basal levels of DHT-untreated cells (AR/Lamin = 0.99). SB216763 induced nuclear export of the AR could be completely inhibited by the CRM1 inhibitor LMB (AR/Lamin = 1.94) (Fig. 4). These results are in perfect agreement and support the observations previously made in PC3 cells transfected with the wild-type AR-EosFP (Fig. 3).

In contrast to the export of endogenous AR as well as transiently transfected AR-EosFP, the nuclear localization of ARΔLBD remained unaffected by GSK-3 inhibition (Fig. 4). Additional experiments with another truncated AR mutant ($AR^{Q640X}$) yielded similar results (data not shown). Based on these observations we hypothesized that the C-terminal end of the AR is necessary for a CRM1 mediated nuclear export of the receptor.

## A CRM1-BINDING SITE IS LOCATED AT THE C-TERMINAL END OF THE AR

In order to be transported out from the nucleus via the CRM1/exportin-1 system, proteins must contain a specific leucine-rich NES that is recognized by the export receptor CRM1. To identify the functional domains of the AR that interact with CRM1, we performed an IP analysis on whole cell lysates from 22Rv1 cells (Fig. 5A). As seen above, 22Rv1 express a full length as well as an LBD-lacking AR isoform. When analyzing the CRM1 co-immunoprecipitates with AR antibodies directed against the C-terminal (EP670Y) or N-terminal (AR441) part of the AR only the full length AR was detectable (Fig. 5A). The absence of the ARΔLBD signal at

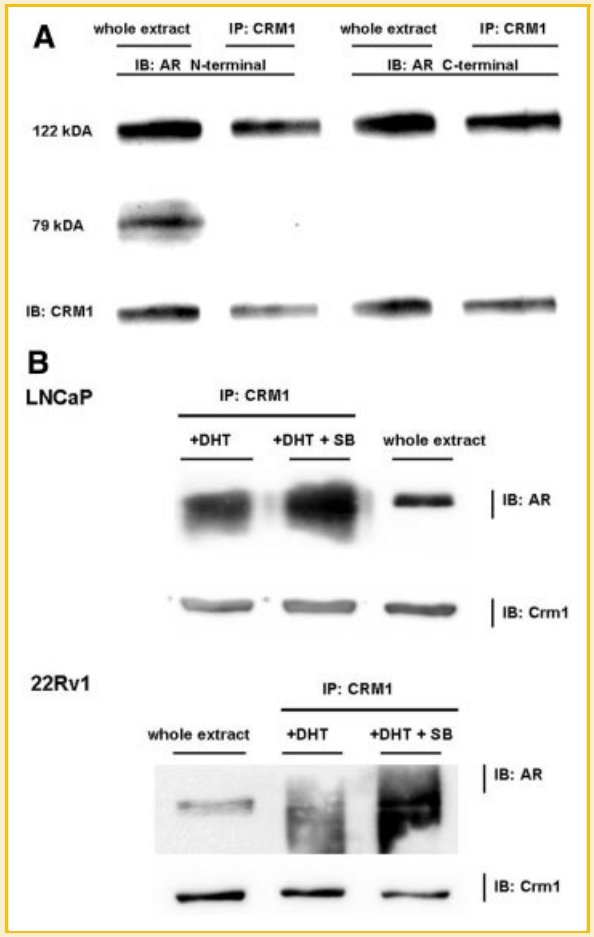


Fig. 5. AR binds to CRM1. (A): 22Rv1 cells were cultured under normal conditions (10% FBS) in T25 flasks for 24 h and proteins were collected. One hundred micrograms of total protein extract was immunoprecipitated with 1 μg CRM1 antibody. Immunoprecipitates were analyzed by monoclonal antibodies AR441 and EP670Y recognizing the N-terminal or the C-terminal end of the AR protein. (B) LNCaP and 22Rv1 cells were cultured under normal conditions in T25 flasks for 24 h. Subsequently cells were incubated in the presence/absence of 1 μM SB216763. Proteins were collected 24 h later. One hundred micrograms of total protein extract was immunoprecipitated with 1 μg CRM1 antibody. Immunoprecipitates were analyzed using AR antibody (AR441).

79 kDa in the immunoprecipitates performed with an antibody directed against the N-terminus of the AR suggests that a putative CRM1 binding site is located at the C-terminal end of the AR. Moreover, based on the translocation experiments with $AR^{Q640X}$ we conclude that the putative CRM1 binding site is located in the LBD between amino acid 640–919 of the AR.

### INHIBITION OF GSK-3*B* INCREASES CRM1/AR INTERACTION

The finding that inhibition of GSK-3β induced a rapid CRM1 mediated nuclear export of the AR in PCa-cell lines prompted us to study the interaction between AR and CRM1 in the presence/absence of the GSK-3 inhibitor SB216763. As shown by co-IP analysis of whole cell lysates from LNCaP cells, there is an increased interaction of the AR with CRM1 in SB216763 treated cells in comparison to untreated control cells (Fig. 5B). In 22Rv1 cells the interaction between CRM1 and the AR following GSK-3-inhibition is even more pronounced than in LNCaP cells (Fig. 5B).

### INHIBITION OF SB216763-INDUCED NUCLEAR EXPORT BY LMB RESTORES AR SIGNALING

As shown previously, inhibition of GSK-3 activity diminishes AR transactivation [Mazor et al., 2004; Rinnab et al., 2008]. To test whether the inhibition of AR transactivation by SB216763 could be rescued by LMB, 22Rv1 cells were subject to DHT treatment in an androgen-dependent reporter gene assay. Treatment of 22Rv1 cells with 10 nM DHT for 5 h almost doubled AR transactivation compared to untreated controls (Fig. 6). Transcriptional activity of AR was significantly lower in cells additionally treated with SB216763 (130% in DHT/SB216763-treated cells vs. 182% in DHT treated cells). The inhibition of AR transactivation by the GSK-3 inhibitor SB216763 could be completely reversed by pre-treatment with LMB (211% in DHT/SB216763/LMB-treated cells vs. 130% in DHT/SB216763-treated cells) (Fig. 6). Taken together this experiment clearly demonstrates that inhibition of the CRM1-mediated export machinery, leads to enhanced AR signaling in human PCa cells.

## DISCUSSION

Prostate cancer is the prototype of an androgen-dependent tumor. In the absence of androgens, the AR predominantly resides in the cytoplasm. Upon ligand binding, the receptor rapidly shuttles into the nucleus. Conversely, in advanced HRPCa cell lines, the ability to

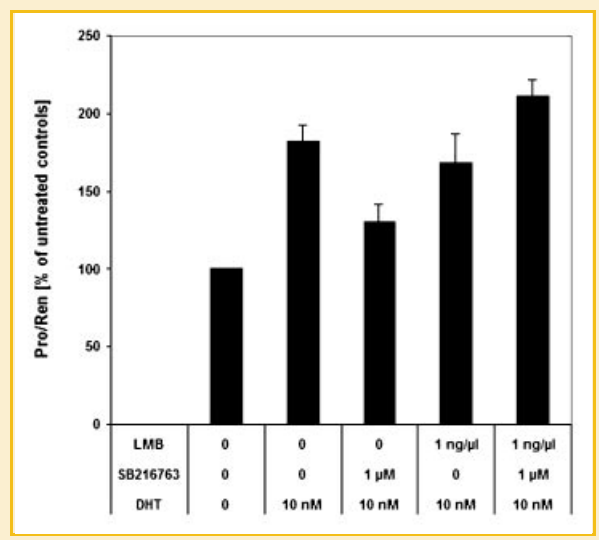


Fig. 6. LMB rescues AR-transcriptional activity in 22Rv1 treated with SB215673. AR-positive 22Rv1 cells were transfected with Probasin and Renilla luciferase reporter plasmids and incubated with 1 ng/μl CRM1-inhibitor LMB for 30 min, followed by DHT-treatment (10 nM) for another 30 min. Cells were treated with 1 μM SB216763 for 5 h. AR transcriptional activity was measured by a reporter gene assay as described in the Materials and Methods Section. Results are expressed as a percent of untreated controls ± SD.

proliferate under sub-physiological levels of androgen has been associated with increased nuclear localization of the AR even in the absence of androgens [Gregory et al., 1998, 2001; Saporita et al., 2007].

Various studies have offered insights into the nuclear import of the AR. A bipartite nuclear localization sequence (NLS) has been identified in the DNA binding domain and hinge region of the AR [Zhou et al., 1994]. This NLS utilizes the classical importin pathway for transport through the nuclear pore complex [Savory et al., 1999; Freedman and Yamamoto, 2004]. In addition, a less defined NLS is present in the LBD [Jenster et al., 1992; Poukka et al., 2000]. Although the mechanism responsible for the nuclear import of steroid receptors is well documented, the mechanisms of AR export remain largely unknown [Saporita et al., 2003; Shank et al., 2008; Nguyen et al., 2009]. In this paper, we demonstrate that GSK-3β, a multifunctional serine/threonin kinase interacts with the AR (Fig. 1) thereby phosphorylating the receptor on serine and threonine residues (Fig. 2). Most interestingly, pharmacological inhibition of GSK-3β activity targets the activated AR for a rapid export from the nucleus thereby down-regulating AR transactivation in human PCa cells (Figs. 3, 4, and 6). The nuclear export following GSK-3 inhibition could be inhibited by LMB, the latter suggesting a CRM1-dependent mechanism (Figs. 3 and 4).

As shown previously, there is a physical interaction of GSK-3β and the AR [Mazor et al., 2004; Salas et al., 2004]. Based on IP studies, we demonstrate that GSK-3β binds the AR both in the absence and presence of androgens and co-immunoprecipitates are detectable in the cytoplasm as well as in the nucleus (Fig. 1). As the detection of co-immunoprecipitates is very strong in the cytoplasm in the absence of androgens as well as in the nucleus upon androgenic stimulation (Fig. 1), a possible co-transport of AR and GSK-3 could be assumed. Although the precise binding site for GSK-3β on the AR has still to be determined, there is evidence that GSK-3β binds to the N-terminal as well as to the C-terminal part of the AR [Salas et al., 2004]. Inhibition of GSK-3 activity by the maleimide SB216763 triggered a rapid nuclear export of endogenous AR as well as transiently over-expressed green fluorescent AR-EosFP in 22Rv1 and PC3 cells (Figs. 3 and 4). Similar results were obtained when incubating AR-EosFP-expressing cells with the aminopyrazole GSK-3 inhibitor XIII and the chloromethyl-thienyl-ketone GSK-3 inhibitor VI (Fig. 3B). In all cases, the nuclear export of the AR protein induced by SB216763 and GSK-3 inhibitor XIII could be rescued by LMB (Fig. 3B). In contrast to the maleimide SB216763 that has been shown to inhibit the activating phosphorylation site tyrosine 216 of GSK-3 [Lochhead et al., 2006], the aminopyrazole moiety of GSK-3 inhibitor XIII blocks the ATP-binding site of GSK-3 [Pierce et al., 2005]. However, both inhibitors operate in an ATP-competitive manner [Meijer et al., 2004]. Therefore we used GSK-3 inhibitor VI, a non-ATP competitive GSK-3β whose inhibitory activity is related to the irreversible binding to a key cysteine residue present in the ATP-binding site of GSK-3, which is relevant for modulation of GSK-3 activity [Perez et al., 2009]. Like SB216763 and GSK-3 inhibitor XIII, GSK-3 inhibitor VI was able to dramatically enhance the nuclear export of AR-EosFP in PC-3 cells (Fig. 3B). This export could be inhibited by pre-incubation of the cells with LMB.

Other non-ATP dependent modes of GSK-3 inhibition like GSK-3 silencing or over-expression of kinase dead GSK-3 were taken into consideration, but were not used for the following reasons: (1) In contrast to the fast acting pharmacological GSK-3 inhibitors, GSK-3 silencing is too slow to analyze nuclear export in real time. An increase in cytoplasmic AR after long-term treatment with siRNA/shRNA does not allow to discriminate between a reduced nuclear import or an increased export of the receptor protein. (2) The use of dominant negative (kinase dead) GSK-3 constructs was not an option as over-expression of kinase dead or dominant negative GSK-3 constructs was shown to impede nuclear entry of GSK-3 [Meares and Jope, 2007]. Changes in the nuclear/cytoplasmic GSK-3 ratio may have unpredictable side effects on the AR localization as AR and GSK-3 interact and eventually co-migrate into the nucleus. In consequence we focused on the inhibition of GSK-3 by structurally and mechanistically different GSK-3 inhibitors. To summarize, all inhibitors triggered a rapid nuclear export of endogenous as well as transiently over-expressed AR-EosFP in 22Rv1 and PC3 cells. Nuclear export of the AR following GSK-3 inhibition was abrogated by LMB, an inhibitor of the CRM1/exportin-1 system (Figs. 3B and 4).

CRM1 is one of the best characterized receptors responsible for the nuclear export of proteins expressing a leucine-rich NES. In order to induce nuclear export, CRM1 has to build a trimeric complex with RanGTP and an NES-containing protein [Liu and DeFranco, 2000]. The interaction of CRM1 with LMB selectively abolishes its ability to bind NES in proteins [Kudo et al., 1999]. Although it is generally accepted that most steroid receptors lack a prototypical leucine-rich NES, there is experimental evidence that steroid receptors can be exported through a CRM1-dependent mechanism [Rimler et al., 2001, 2002; Itoh et al., 2002]. In agreement with these observations, an NES with limited homology to the canonical CRM1 site was recently found in the C-terminal end of the estrogen receptor [Lombardi et al., 2008].

The assumption that a functional putative CRM1 binding site is also located in the LBD of the AR is supported by co-IP analysis of CRM1 with different AR isoforms (Fig. 4A). 22Rv1 are known to express two AR isoforms, a mutated and extended AR (122 kDa) and a C-terminally deleted 79 kDa isoform termed ARΔLBD. When analyzing the CRM1 immunoprecipitates with antibodies recognizing the N/C-terminal end of the AR, only the full-length AR, but not the ARΔLBD isoform, was detectable (Fig. 4A). This observation clearly shows that CRM1 interacts with a sequence located in the C-terminal end of the AR. In addition, treatment of LNCaP as well as 22Rv1 cells with SB216763 enhanced CRM1-binding to the AR in both cell lines, although CRM1/AR binding was more pronounced in 22Rv1 as compared to LNCaP cells (Fig. 5B). This difference could be due to an inactivating Serine 9-phosphorylation of GSK-3 in LNCaP reported by several authors [Mazor et al., 2004; Salas et al., 2004]. However, GSK-3 activity is not fully inhibited in LNCaP. There is experimental evidence that the extent of GSK-Ser9 phosphorylation in LNCaP can still be regulated leading to a modulation of various physiological functions [Liao et al., 2003; Kumar et al., 2004; Hartel et al., 2003; Liu et al., 2008; Rinnab et al., 2008, Supplemental Data].

Based on these results we postulate that a functional GSK-3 dependent CRM1-binding site is located in the LBD of the AR.

Although we did not identify the exact localization of this putative $NES^{CRM1}$ on the AR, it functionally differs from the leptomycin-insensitive $NES^{AR}$, recently identified by Saporita et al. (2003). Under these circumstances, it is interesting to note that androgen withdrawal, in contrast to GSK-3β inhibition, causes a slow and leptomycin-insensitive nuclear export of the AR (Supplemental Data) [Poukka et al., 2000; Tyagi et al., 2000; Saporita et al., 2003]. A similar phenomenom was described for the glucocorticoid receptor (GR). As shown by Itoh et al. [2002], UV-induced nuclear export of the GR is dependent on the activity of the c-JUN N-terminal kinase (JNK), a stress inducible mitogen activated protein kinase. The UV/JNK-induced rapid export of the GR can be inhibited by LMB, indicating that CRM1 plays a pivotal role in stress-induced nuclear export of the GR. In contrast to these findings, nuclear export of the GR induced by dexamethasone-withdrawal is relatively slow and remains unaffected by LMB treatment [Itoh et al., 2002]. In summary the AR as well as the GR studies suggest that steroid receptors may be targeted to different export systems depending on the physiological conditions. Therefore, the mechanism by which GSK-3β interferes with the CRM1 machinery needs to be elucidated.

AR transcriptional function depends on the nuclear localization of the receptor. In HRPCa cells, a considerable great amount of AR is located in the cell nucleus even upon androgen ablation [Gregory et al., 1998, 2001]. The predominant nuclear localization of AR in HRPCa requires mechanisms directing ligand-independent nuclear import and/or means of retaining AR in the nucleus in the absence of androgens. As shown in this study, inhibition of GSK-3β activity leads to a rapid CRM1-dependent nuclear export of the AR. Once in the cytoplasm, the AR is prone to proteosomal degradation [Rinnab et al., 2008]. Defining enzymes that affect components of the AR export machinery could provide new strategies for the therapy of advanced HRPCa.

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