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14-3-3 sigma increases the transcriptional activity of the androgen receptor in the absence of androgens

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Abstract

The androgen receptor (AR) is a ligand-activated transcription factor that regulates numerous target genes, including prostate specific antigen (PSA). We examined the ability of each member of the 14-3-3 family to modulate transcription of PSA through the AR. Despite significant homology within the 14-3-3 family we observed differences in the ability of each isoform to alter the transcriptional activity of the AR. Significantly, 14-3-3 sigma activated PSA-luciferase reporters not only at castrate levels of androgens, but also in the complete absence of androgens. 14-3-3 sigma also increased expression of the endogenous PSA gene in the absence of androgens. Knockdown of the AR by siRNA oligonucleotides abolished activation of these reporters by 14-3-3 sigma. These findings may have greatest significance in hormone refractory prostate cancer where the AR may be activated in a ligand-independent manner.

Keywords

prostate cancer; 14-3-3 sigma; androgen receptor; transcriptional activator; LNCaP

1. Introduction

Prostate cancer is the second leading cause of cancer death among American men [1]. Organ-confined disease is treatable by means of radical prostatectomy or radiotherapy, but while initially effective, androgen deprivation therapy for metastatic disease is strictly palliative. The eventual emergence of androgen-independent disease leaves patients with few effective treatment options, none of which extend survival by more than a few months. The molecular mechanism of disease progression remains uncertain (see [2,3] for reviews).

The androgen receptor (AR) is a ligand-activated transcription factor required for androgen regulation of genes such as prostate specific antigen (PSA). Increased expression of PSA in the sera of men after androgen deprivation therapy is the hallmark for identifying androgen-independent disease. Numerous other androgen-regulated genes also become re-expressed with progression to androgen independence, implying that the AR is active in these tissues [3,4].

Ligand-independent activation of the AR is suggested to be a significant contributor to this progression to androgen-independent disease [3,5]. One proposed mechanism by which the AR is activated in the absence of androgens is by the action of coregulatory proteins stimulated by other signalling pathways [5,6]. For example, interleukin-6 (IL-6) caused ligand-

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independent activation of the AR through the activity of steroid receptor coactivator 1 (SRC1), the mitogen-activated protein kinase (MAPK) pathway, and signal transducer and activator of transcription 3 (STAT3) [7,8]. Increased expression and/or activity of these proteins has also been correlated with prostate cancer and the development of androgen independence [9-12]. Additionally, the coactivator p300 was recently suggested to activate the expression of PSA independent of the AR in LNCaP cells after long term treatment with IL-6 [13].

The 14-3-3 family of proteins regulate a large number of cellular processes, including apoptosis, response to DNA damage, and mitogenic signalling [14]. Members of this family form homo- and heterodimers, which can then bind to a diverse array of target proteins. Binding of 14-3-3s to target proteins modulates the function of those targets by, for example, altering their subcellular localization or protein interaction partners. This family consists of 7 different isoforms that share a high degree of sequence identity and conservation [15]. Despite this conservation, numerous isoform-specific observations have been described, including differences in binding partners, subcellular localization, and structure [16-18]. 14-3-3 eta was previously shown to enhance AR transcriptional activity in the presence of ligand, with no effect seen in the absence of ligand [19]. Unfortunately, the effects of other members of this protein family were not reported. Therefore, we investigated the effect(s) of all seven members of the 14-3-3 family of proteins on the transcriptional activity of the AR in prostate cancer cells. We demonstrated that 14-3-3 sigma specifically increased the transcriptional activity of the AR, particularly in the absence of androgens. To our knowledge, this is the first report to examine the effects of all seven 14-3-3 isoforms in the same system under identical conditions.

2. Materials and Methods

2.1. Cell culture

LNCaP cells obtained from Dr. L.W.K. Chung (Emory University School of Medicine, Atlanta, GA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/mL penicillin, and 100 µg/µL streptomycin. All chemicals were purchased from Sigma, unless stated otherwise. Androgen stimulation was performed by treatment of cells with the synthetic androgen R1881 (PerkinElmer, Woodbridge, Canada).

2.2. Plasmids

The ARR₃-tk-luciferase reporter plasmid has been described previously [20]. PSA (6.1 kb)-luciferase was a gift of Dr. J.-T. Hsieh (University of Southwestern Medical Center, Dallas, TX). The expression vectors for the seven isoforms of 14-3-3 were generously provided by Dr. H. Fu (Emory University School of Medicine, Atlanta, GA) [21]. The control expression vector pDEST26 was generated by removing the *BsrGI* fragment from the 14-3-3 zeta-encoding plasmid, eliminating the entire coding sequence. All plasmids were sequence verified.

2.3. Transfection and luciferase assay

LNCaP cells were plated at 3.0×10^5 cells per well in 6-well plates. After 24 hr, transfections were performed in serum-free media using LIPOFECTIN® Reagent (Invitrogen) according to the manufacturer's protocol. Each 14-3-3 expression vector was transfected at 500 ng/well unless indicated otherwise, and each luciferase reporter construct was transfected at 1.0 µg/well. The total amount of transfected DNA was normalized to 3 µg/well by the addition of a promoterless plasmid [7,8]. After 24 hr serum-free RPMI 1640 containing R1881 (0.001 - 10 nM), or an equal amount of vehicle (ethanol), was added to the cells. Cell lysates were harvested after 24 or 48 hr incubation using Passive Lysis Buffer (Promega, Nepean, Canada). Luciferase activities were measured on a multiplate luminometer (EG & G Berthold, Germany) using the Dual Luciferase Assay System (Promega). Luciferase activities were normalized to the protein

concentration of the lysates [7,22] as determined by Bradford assays [23]. All transfection experiments were performed in triplicate wells and repeated at least 3 times. The results are presented as fold induction relative to pDest26 alone.

2.4. Real-time PCR

LNCaP cells stably transfected with 14-3-3 sigma or pDest26 were treated for 16 hr with 10 nM R1881, or an equal amount of vehicle (ethanol), before harvesting total RNA using TRIZOL® Reagent according to the manufacturer's protocol. Genomic DNA was degraded using the DNA-free™ kit (Ambion) according to the manufacturer's protocol prior to reverse transcription using SuperScript® III enzyme (Invitrogen). Quantitative real-time PCR was performed on an ABI 7900 thermocycler using the Platinum® SYBR® Green qPCR SuperMix kit with uracil-N-glycosylase (UDG) and ROX reference dye (Invitrogen). The primers used were: PSA-forward, CCAAGTTCATGCTGTGTGCT; PSA-reverse, CCCATGACGTGATACCTTGA; GAPDH-forward, CTGACTTCAACAGCGACACC; and GAPDH-reverse, TGCTGTAGCCAAATTCGTTG. Triplicate PCR reactions were performed for each of three independent experiments, and the average normalized expression is shown.

2.5. AR siRNA

A 21-nucleotide double-stranded siRNA duplex targeting nucleotides 293-312 (5'-aagcccatcgtagaggcccca-3') of the AR was obtained from Dharmacon Research (Boulder, CO). A control double-stranded siRNA (AR-SCR) targeting the inverse sequence of nucleotides 293-315 (5'-accccgagatgctaccgaa-3') of the AR was also generated. The efficacy of these molecules was shown previously [24]. LNCaP cells were plated at 2.0×10^5 cells per well in 6-well plates and incubated in RPMI 1640 with 5% FBS for 24 hr. Cells were fed fresh serum-free RPMI 1640 and transfected with 100 nM of siRNA duplex using OLIGOFECTAMINE® Reagent (Invitrogen) according to the manufacturer's protocol. After 24 hr the cells were incubated for a further 24 hr in the presence of 5% FBS prior to transfection with the luciferase reporters (1.0 µg/well) and 14-3-3 isoforms (1.0 µg/well) in serum-free media as described above. Again, the total amount of DNA transfected was normalized to 3 µg/well by the addition of a promoterless plasmid.

2.6. Western blot analysis

LNCaP cells were grown and treated in parallel with the siRNA-transfected cells analyzed for luciferase activity. These cells were harvested and washed twice in ice-cold PBS. The cell pellets were lysed in 2x SDS-PAGE loading buffer and boiled for five minutes prior to loading on 12% SDS-PAGE gels. Alternatively, cell lysates from the luciferase assays were also used for western blotting. The separated proteins were transferred to nitrocellulose membranes and blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for one hour at room temperature or at 4°C overnight, and then washed three times. The blots were probed with anti-AR (441)(1:500) or anti-His (H-15)(1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 to 2 hours at room temperature, washed three times, and incubated with the appropriate IR-labelled secondary antibody (1:5000, Molecular Probes) for 30 minutes at room temperature. All washes were performed with PBS containing 0.1% Tween20. Primary antibodies were diluted in Odyssey Blocking Buffer with 0.1% Tween20, while secondary antibody dilutions contained an additional 0.01% SDS. Visualization was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences).

3. Results

3.1. 14-3-3 sigma specifically enhanced AR transcriptional activity and PSA expression

To examine the effects of all the 14-3-3 isoforms on the transcriptional activity of the AR we utilized two different luciferase reporter constructs. The PSA (6.1 kb)-luciferase reporter construct contains both the enhancer and promoter regions of the human PSA locus and is highly sensitive to androgens [25]. The second construct, ARR₃-thymidine kinase (tk)-luciferase, is an artificial reporter containing three tandem repeats of the ARE1 and ARE2 regions of the rat probasin gene [20]. Both of these reporters were transiently transfected into LNCaP cells along with each isoform of 14-3-3, or the empty vector pDest26, and then treated with the synthetic androgen R1881 (10 nM) or its carrier, ethanol (0.00055 % v/v). Both reporter constructs displayed similar fold induction trends for each 14-3-3 isoform when compared to the empty vector (Fig. 1A,B). LNCaP cells treated with R1881 showed small inductions of reporter activity by the various isoforms of 14-3-3 relative to the empty vector (Fig. 1A). One-way analysis of variance (ANOVA) followed by a multiple comparison test determined that both the sigma (2.2-fold) and tau (2.0-fold) isoforms caused a statistically significant increase ($p < 0.01$) in PSA (6.1 kb)-luciferase reporter activity relative to the empty vector. Surprisingly, though, 14-3-3 sigma was also found to greatly increase the activity of both reporters in the absence of androgens (Fig. 1B; $p < 0.001$). This effect was specific to the sigma isoform as no other isoform significantly increased the activity of either reporter construct in the absence of androgens. Western blotting confirmed that all the isoforms were expressed in these cells (Fig. 1C). Together, these data indicate that the 14-3-3 sigma protein is uniquely able to increase the transcriptional activity of the AR, especially in the absence of androgens.

We also tested the ability of 14-3-3 sigma to alter expression of the endogenous PSA gene. LNCaP cells were generated that stably expressed either 14-3-3 sigma or the empty vector pDest26. These cells were treated with R1881 (10 nM) or its vehicle (ethanol) and PSA expression was measured using quantitative real-time PCR (Fig. 1D). In the presence of androgen, the expression of 14-3-3 sigma had no effect on PSA expression. In the absence of androgens, though, the presence of 14-3-3 sigma protein resulted in a 2.1-fold increase ($p = 0.002$) in PSA expression. This finding confirmed that 14-3-3 sigma could increase AR transcriptional activity within the native chromatin structure of the PSA locus.

3.2. 14-3-3 sigma enhanced AR activity in a dose-dependent manner in the absence of androgens

We next examined the effect of increasing amounts of 14-3-3 sigma expression on reporter activity. All levels of 14-3-3 sigma caused a small, but consistent increase in reporter activity when cells were treated with R1881 (Fig. 2A). In contrast, reporter activity increased in a dose-dependent manner when the cells were grown in the absence of androgens (Fig. 2B). This suggests that only small amounts of 14-3-3 sigma protein were sufficient to activate AR-dependent reporters, but in the absence of androgens increasing amounts of this isoform were able to further increase reporter activity.

3.3. 14-3-3 sigma increased the sensitivity of AR to low levels of androgens

Since 14-3-3 sigma strongly activated these reporter constructs in the absence of androgens, we investigated the effect of the sigma isoform at the low levels of androgens frequently observed in patients undergoing androgen deprivation therapy. To test this, a range of R1881 concentrations (0.001 - 10 nM) were used. While the absolute levels of luciferase activity increased with increasing R1881, the degree of induction caused by 14-3-3 sigma was actually greatest at low concentrations of R1881 (Fig. 3). These results implicate a mechanism whereby 14-3-3 sigma is able to sensitize the AR to low levels of androgen.

3.4. Induction of PSA reporters by 14-3-3 sigma was dependent on AR

To determine whether the activation of these reporter constructs by 14-3-3 sigma was mediated through the AR, a siRNA oligonucleotide strategy targeting the AR was used to reduce the amount of AR protein prior to the expression of 14-3-3 sigma in these cells. Consistent with our previous results, cells transfected with a control scrambled oligonucleotide (AR-SCR) and 14-3-3 sigma showed a statistically significant increase in reporter activity in both the presence and absence of androgens (Fig. 4A,B; $p < 0.02$). Transfection with an AR-specific siRNA oligonucleotide (AR-siRNA) greatly reduced the expression of AR protein in these cells (Fig. 4C). As expected, knock-down of AR protein levels led to decreased reporter activity regardless of the presence of 14-3-3 sigma or treatment with androgens. Significantly, though, expression of 14-3-3 sigma in AR-siRNA-transfected cells did not result in increased reporter activity (Fig. 4A,B). These data suggest that the AR was required for 14-3-3 sigma to activate this reporter construct.

3.5. 14-3-3 sigma did not alter AR protein levels

We next investigated possible mechanism(s) by which 14-3-3 sigma could increase AR transcriptional activity. We first sought to determine if 14-3-3 sigma could bind directly to the AR to alter its activity, as was previously observed for the glucocorticoid receptor (GR), a related steroid nuclear receptor [26]. However, we did not detect a direct interaction between these proteins by co-immunoprecipitation under the conditions tested (data not shown). 14-3-3 sigma was also previously shown to inhibit the transcriptional activity of GR by sequestering the receptor in the cytosol [26]. Therefore, we monitored the subcellular localization of a green fluorescent protein (GFP)-AR fusion protein in the presence and absence of 14-3-3 sigma. No difference in AR localization was observed in the presence or absence of androgens when compared to cells transfected with empty vector alone (data not shown). Finally, it was recently shown that expression of 14-3-3 eta increased GR protein levels by inhibiting degradation of the receptor, which led to increased transcriptional activity of the GR [27]. However, western blot analysis demonstrated that the presence of 14-3-3 sigma did not alter the level of AR protein in these cells (Fig. 5). Thus, while the AR must be present for 14-3-3 sigma to activate these reporter constructs, the expression of 14-3-3 sigma does not increase AR activity by simply increasing the amount of AR protein present in the cell or by significantly altering the subcellular localization of the AR.

4. Discussion

Members of the 14-3-3 protein family are involved in regulating a wide range of cellular events. Various isoforms of this family have also been previously shown to regulate the transcriptional activity of their target proteins, including steroid nuclear receptors [14,19,28]. However, the studies investigating the AR and GR have focused on individual isoforms and the experiments have been performed in a variety of cell types. This is the first report to examine the effects of all seven isoforms on the transcriptional activity of the AR in a single system. We have shown that 14-3-3 sigma, in contrast to the other family members, greatly enhanced the transcriptional activity of the AR in the absence of androgens.

In contrast to our findings with the AR, Kino, et al. [26] demonstrated that 14-3-3 sigma inhibited the transcriptional activity of the GR by sequestering the receptor in the cytosol. Similarly, 14-3-3 eta was shown to enhance GR activity via two different mechanisms. This isoform was shown to alter the subcellular localization of the GR corepressor RIP140 and increase the amount of GR protein, with both mechanisms resulting in increased GR transcriptional activity [27,29]. Finally, 14-3-3 eta was also shown to increase the transcriptional activity of the AR, but only in the presence of androgens, and the mechanism by which this occurred was not examined [19]. Our results showed minimal induction of AR

activity in the presence of 14-3-3 eta expression, but this difference in results is likely due to differences in the way these experiments were performed. Our experiments utilized the LNCaP cell line, which expresses an endogenous AR, and the PSA (6.1 kb) and ARR₃-thymidine kinase (tk)-luciferase reporters, which are well characterized androgen-regulated reporter constructs. In contrast, the experiments of Haendler et al. [19] were performed in PC3 prostate cancer cells stably transfected with the AR and employed a novel androgen-regulated reporter based on the CRISP-1 promoter.

Previous studies have focused mainly on activity of the receptor in the presence of ligand, and little to no increase in transcriptional activity was seen in the absence of ligand [19]. In contrast, our experiments highlight the unique ability of 14-3-3 sigma to enhance AR transcriptional activity in the absence of androgens. Consequently, our work suggests that 14-3-3 sigma may play an important role in sensitizing the AR to low levels of androgens. These findings may be most relevant in the setting of androgen independent prostate cancer. Androgen withdrawal therapy results in decreased, but detectable, levels of dihydrotestosterone in the prostate [30]; the expression of 14-3-3 sigma may enable the AR to still respond strongly to these castrate levels of androgen. The increase in endogenous PSA expression in LNCaP cells stably expressing 14-3-3 sigma is also more representative of the clinical scenario. This experiment showed that long-term expression of 14-3-3 sigma resulted in greater AR transcriptional activity, further suggesting that tumours constitutively expressing 14-3-3 sigma protein may be more sensitive to low levels of androgen.

We investigated a number of mechanisms by which 14-3-3 sigma could activate the AR. Direct protein-protein interactions were previously demonstrated between 14-3-3 sigma and GR [26] as well as between 14-3-3 eta and both the GR and the AR [28,29]. However, we did not detect a direct interaction between the AR and 14-3-3 sigma in our co-immunoprecipitation experiments. Our results also suggest that expression of 14-3-3 sigma did not alter the amount or subcellular localization of AR protein. One potential mechanism yet to be investigated is whether 14-3-3 sigma may alter the amount of, or localization of, an AR corepressor, leading to increased AR transcriptional activity. A comparable situation was observed for the GR where 14-3-3 eta was able to sequester the GR corepressor RIP140 in the cytosol, thus allowing the GR to have greater activity in the nucleus [29]. While it would be interesting to determine what protein(s) interact with 14-3-3 sigma to mediate its effects on AR, 14-3-3 sigma has been demonstrated to interact with hundreds of proteins [31]. It is therefore difficult to speculate which of these interactions may be responsible for increasing AR activity.

14-3-3 sigma appears to have a number of characteristics unique from the other 14-3-3 isoforms. For example, 14-3-3 sigma tends to only form homodimers while other 14-3-3 isoforms readily form heterodimers with multiple other isoforms [18]. The determination of the crystal structure of 14-3-3 sigma revealed a structural basis for this finding [18,32]. Various other isoform-specific functions have also been described, for instance in the subcellular localization of the sigma and zeta isoforms [17]. Additionally, all other isoforms of this family were shown to bind the nuclear localization sequence of p27, but only 14-3-3 sigma was unable to bind this region [33]. Finally, Cdc25C was also shown to be bound by other 14-3-3 isoforms, but not sigma [16,34]. Structural analyses revealed a secondary surface on 14-3-3 sigma that influenced the specificity of its interacting partners [18]. This region contained three residues perfectly conserved in all isoforms except sigma. Mutation of these residues to the consensus sequence of the other isoforms enabled 14-3-3 sigma to interact with Cdc25C [18], revealing a potential mechanism by which 14-3-3 sigma is able to exert specific effects on target proteins. Thus, it is not unexpected that 14-3-3 sigma was uniquely able to alter the transcriptional activity of the AR in our experiments.

Loss of 14-3-3 sigma has recently been correlated with the development of prostatic intraepithelial neoplasia, and the majority of patients with invasive prostate cancer displayed low or no expression of this isoform [35-37]. This decreased expression is due to promoter hypermethylation in both primary prostate cancer samples and in the LNCaP cell line [36, 38]. However, these studies also showed that a subset of patients appeared to retain 14-3-3 sigma expression. Significantly, Cheng et al. [35] also observed an increased frequency of expression of 14-3-3 sigma in the more poorly differentiated samples characterized by higher Gleason scores. Huang et al. [39] correlated increased nuclear localization of 14-3-3 sigma with decreasing differentiation of tumours. Consistent with this, strong expression of 14-3-3 sigma was observed in the androgen-independent prostate cancer cell lines PC3, DU145, and 22Rv1, and expression of 14-3-3 sigma led to increased chemoresistance [36,38,40]. Increased expression of 14-3-3 sigma was also observed in response to treatment with androgens, and a reporter containing the 14-3-3 sigma promoter was induced within three hours of androgen treatment [39]. Previous studies have suggested that genes regulated by the AR are re-expressed in androgen independent disease [4]. It will be interesting to examine the expression of 14-3-3 sigma in androgen-independent tissues as the presence of 14-3-3 sigma protein may lead to increased activation of the AR in a subset of patients with androgen independent prostate cancer.

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Abbreviations

AR, androgen receptor; GR, glucocorticoid receptor; PSA, prostate specific antigen.

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Fig 1.

The transcriptional activity of the AR was activated specifically by 14-3-3 sigma. LNCaP cells were transfected with each 14-3-3 isoform or the empty vector pDest26 and either the PSA (6.1 kb)-luciferase or the ARR₃-tk-luciferase reporter construct, followed by treatment with either 10 nM R1881 (A) or vehicle (B). The results are presented as fold induction relative to empty vector alone, and the error bars represent the standard deviation of the mean of three independent experiments. *, $p < 0.001$ as determined by one way ANOVA and Tukey's multiple comparison test. (C) Immunoblot demonstrating expression of each His-tagged 14-3-3 isoform in transfected cells. Probing for β -actin confirmed the amount and quality of protein in each lane. (D) Real-time PCR analysis of endogenous PSA expression in LNCaP cells stably

expressing 14-3-3 sigma or pDest26. PSA transcript levels were normalized to GAPDH expression, and fold-induction in the presence of 14-3-3 sigma is shown. *, $p=0.002$ as determined by Student's t-test.

Fig 2.

Increasing amounts of 14-3-3 sigma increased the level of AR activity in the absence of androgens. LNCaP cells were transfected with ARR₃-tk-luciferase and the indicated amounts of 14-3-3 sigma (σ), and the total amount of DNA transfected was kept equal with the addition of pDest26 empty vector. The cells were then treated with either 10 nM R1881 (A) or vehicle (B). The results are presented as fold induction relative to empty vector alone, and the error bars represent the standard deviation of the mean of three independent experiments. All transfections and treatments were performed in the absence of serum.

Fig 3.

14-3-3 sigma sensitized the AR to low levels of androgen. LNCaP cells transfected with equal amounts of 14-3-3 sigma or pDest26 empty vector were treated for 48 hours with the indicated concentrations of R1881 prior to measuring reporter activity. Three independent experiments were performed in triplicate for each treatment, and representative results are shown. Error bars represent the standard deviation of the mean of triplicate wells. All transfections and treatments were performed in the absence of serum.

Fig 4.

Activation of the ARR₃-tk-luciferase reporter construct by 14-3-3 sigma was dependent on the AR. LNCaP cells were transfected with an siRNA oligonucleotide targeting the AR (ARsiRNA) or a scrambled oligonucleotide (AR-SCR) prior to transfection with 14-3-3 sigma (σ) or pDest26 empty vector. The cells were then treated with 10 nM R1881 (A) or vehicle (B). Activity of the luciferase reporter is expressed as fold induction relative to empty vector plus AR-SCR oligonucleotide, and the error bars represent the standard deviation of the mean of three independent experiments. * $p < 0.02$ as determined by Student's t-test between cells treated with R1881 and vehicle in the presence of the AR-SCR control oligonucleotide. (C) Immunoblot demonstrating an efficient decrease in AR protein levels in the presence of the

AR-siRNA oligonucleotide under both treatment conditions. Probing for β -actin confirmed the amount and quality of protein in each lane. All transfections and treatments were performed in the absence of serum.

Fig 5.

Expression of 14-3-3 sigma did not alter the amount of AR protein in the cells. LNCaP cells were transfected with either 14-3-3 sigma (σ), 14-3-3 epsilon (ϵ), or pDest26 empty vector and treated with 10 nM R1881 or vehicle for 48 hours. All transfections and treatments were performed in the absence of serum. Whole cell lysates were harvested and immunoblotted with an antibody specific for the AR. Probing for β -actin confirmed the amount and quality of protein in each lane.