Influence of Short Polyglutamine Tracts and p160 Coactivators on the Transactivation of the Androgen Receptor

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Abstract

The androgen receptor (AR) acting as a transcription factor plays a pivotal role in the occurrence and progression of prostate cancer (CaP). Several AR-related factors or modulators have been reported to influence AR activity. Whether and how these factors cooperatively modulate the AR activity has not been well defined. In the present study, the combined effect of p160 coactivators, short CAG length (encoding a short polyQ tract), and AR mutations on AR transactivation in a yeast system was evaluated. It was found that the short polyQ tract can upregulate the transactivation of the wild-type (WT) AR and partial-function (PF) AR mutants in response to a physiological level (10^{-9} M) of dihydrotestosterone. Addition of a p160 coactivator (SRC-1 or TIF2) to the above systems resulted in a significant increase in the ligand-stimulated transactivation. Although the androgen antagonist bicalutamide could suppress the activity of androgen-activated WT or PF ARs, it was unable to do so for gain-of-function AR mutants. A combination of the short polyQ tract and coactivator TIF2 acted cooperatively on the WT AR and PF AR mutants to enhance their transactivation in response to either a low level of dihydrotestosterone (10^{-10} M) or adrenal dehydroepiandrosterone. Taken together, this finding suggests that the modulated AR activity may involve early in the carcinogenesis of CaP. Additionally, these data support the concept that a given CaP in which the AR activity is modulated by multiple AR modulators may progress more readily to castrate resistance.

Key words: androgen receptor, prostate cancer, yeast assay

Introduction

Prostate cancer (CaP) is an extremely heterogeneous tumor.¹ When CaP metastasizes, these patients are customarily treated by some form of androgen ablative therapy (AAT) including monotherapy with an antiandrogen, orchiectomy, or a luteinizing hormone-releasing hormone agonist.² The choice of the AAT is largely dependent on the prescribing preference of the treating physician. For example, although the antiandrogen (bicalutamide [BIC]) may be given for a short period of time at the initiation of luteinizing hormone-releasing hormone therapy, for some patients, a 5- α reductase inhibitor may be added, and this therapy may either be continuously administered for the life of the patient or it may be given on an intermittent basis. Unfortunately, failure of every form of AAT inevitably occurs as judged by an elevated serum PSA level, and subsequently the patient's

tumor becomes castrate resistant (CR), also referred to as hormone refractory or androgen independent. At the CR stage, if a patient has been receiving an antiandrogen, it is discontinued, whereas otherwise such treatment may be initiated. In ~30% of patients, this results in a short-lived PSA response and occasionally in a longer response. When serum PSA increases again, a secondary AAT protocol may be started. This may consist of kenoconazole, diethylstilbestrol, or abiraterone. When these fail, treatment usually proceeds to chemotherapy.^{3,4} The reason that the choice of AAT is so empirical is because the functionality of the androgen receptor (AR) in a given patient's tumor is not known, and, thus, there is no way to test which therapy or combination of therapies would be most effective in any particular case. It is evident, therefore, that improved clinical outcome could depend on a better understanding of how the AR functions in the tumors of individual patients. Efforts to determine the factors that

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influence the behavior of the AR and the extent of their influence may provide important clues to appropriate treatment for individual patients.

The AR acts as a transactivational factor that transduces signaling, conveyed by androgen. Clinically, the AR can be detected in premalignant prostate cells as well as in primary, metastatic, and CR CaPs, suggesting that this receptor contributes to all stages of CaP growth and pathogenesis.^{5,6} The influence of the AR on target genes is modulated by a number of AR-related factors or modulators, such as over-expressed coactivators, short CAG repeat (encoding a polyglutamine or polyQ tract), and somatic mutations of the AR gene. A number of AR coactivators have been identified. Of these coactivators, two p160 family members (SRC-1 and TIF2/SRC-2) are of significant interest in the CR growth of CaP cells. They interact with the AR that has been recruited to AR target genes and act to enhance AR-dependent transcription.⁷⁻⁹ CAG repeat length in exon 1 of the AR gene plays a role for fine-tuning of AR activity.¹⁰ Short CAG repeat lengths have been shown to be associated with an elevated risk of developing CaP, as well as earlier age-of-onset, and more advanced cancer grade and stage at diagnosis.^{11,12} Mutations of the AR gene confer different functionalities on the AR. Studies have shown that approximately half of CaP-derived AR mutations have various extents of gain-of-function (GOF) activities, including promiscuous activity (i.e., allowing activation of the AR by nonandrogenic steroids and/or antiandrogens), whereas one third are partial-function (PF) mutations resulting in the receptor having reduced response to the physiological level of androgens.^{13,14} In clinical CaP, tumor cells often carry more than one AR modulator. Whether and how these factors cooperatively affect AR activity has not been well defined.

Investigators have been attempting to assess complex effect of these AR-related modulators on the AR activity. The first AR mutation that was identified in CaP is a threonine (T) to alanine (A) substitution at position 877 of the AR gene. This results in a promiscuous AR-T877A mutant.¹⁴ A recent study evaluated the influence of CAG repeat lengths and β -catenin coactivator on the AR-T877A and found that CAG repeat length variation and the presence of coactivator cooperatively influenced the transactivational activity of this AR mutant.¹⁵ To date, 88 different point-mutations in the AR gene have been identified in CaP (www.mcgill.ca/androgendb, updated in October, 2008). However, a majority of CaP-derived AR mutations have not yet been studied in this way. One reason for this is that current investigations of AR activity have usually been carried out in mammalian cells. In these cells, there are many endogenous factors that can affect AR function either directly or indirectly. There is, thus, a considerable need for a new analytical model that lacks these intrinsic factors so that they can be introduced experimentally, thereby determining their influence individually and collectively on AR activity.

Saccharomyces cerevisiae is an attractive host model for the analysis of nuclear receptor function.¹⁶ This yeast strain has conserved fundamental processes that are also found in mammalian cells such as transcription initiation and regulation. Further, the AR has been validated to function as a ligand-dependent transactivator in yeast.¹⁷ In a previous study, a colorimetric yeast AR functional assay was described.¹⁴ Although that assay can be used to rapidly com-

pare the transactivational capacities of different AR mutants in response to different ligands, it is not suited for quantitative estimates of AR activity. We modified that yeast assay, adapting it for quantitative measurements of AR transactivation. In this article, this new quantitative yeast assay has been used to investigate the influence of several AR modulators on AR transactivation either singly or in combination. The results of this study show that these modulators can cooperatively modulate the transactivational activity of the AR in response to a low or physiological dose of dihydrotestosterone (DHT), or to adrenal dehydroepiandrosterone (DHEA). It is believed that these results could form the basis of a clinical test, although it is evident that considerable work needs to be done to reach this goal.

Materials and Methods

Construction of plasmids

The yeast plasmids expressing the wild-type (WT) or mutant AR alleles with a normal 25-CAG repeat length and containing the yeast *TRP1* gene as a selectable marker were previously described.¹⁴ In this study, some of these plasmids were engineered to express different AR alleles having either a short 17-CAG or a longer 33-CAG repeat length. These CAG repeat lengths were confirmed by DNA sequencing. 17-, 25-, and 33-CAG repeat lengths were selected for study, as >90% of human AR CAG lengths fall within the range of 16–29-CAG repeats.¹⁸ For construction of p160 coactivator expression vectors, the coding regions of SRC-1 and TIF2 genes were separately released from plasmids pSH-CMV-SRC-1 and pSH-CMV-TIF2 kindly provided by Dr. Hongwu Chen (University of California Davis, School of Medicine). These genes were then cloned into the plasmid pESC-His (Stratagene) downstream to the promoter *GAL1*. These p160 coactivator expression vectors contain the yeast *HIS3* gene as a selectable marker. The luciferase reporter plasmid pXB11-Luc was generated by cloning the firefly luciferase (Luc) gene into the pXB11 plasmid¹⁴ downstream to the yeast *CYC1* minimal promoter where it is under the control of three 26-bp consensus androgen response elements (AREs). All cloned DNA fragments in the constructed plasmids were sequenced to verify the correct reading frames and the absence of random mutations.

Yeast strains and transformation

The yeast *S. cerevisiae* strain BJ2168 (*MATa*, *leu2*, *trp1*, *ura3-52*, *pep4-3*, *prc1-407*, and *prb1-1122*) was a gift from Dr. Didier Picard (University of Geneva, Geneva, Switzerland). To create a luciferase reporter strain, the androgen-responsive reporter plasmid pXB11-Luc was linearized at its unique *ApaI* site in the *URA3* region and integrated into the chromosomal *URA3* locus, resulting in the yARE-Luc reporter yeast strain. The reporter strain was grown on complete YPGA medium (1% yeast extract, 2% bacto-peptone, 3% galactose, and 0.01% adenine). Yeast was transformed with AR-expressing plasmids and/or coactivator-expressing plasmids using the lithium acetate protocol.¹⁴ Yeast transformants were selected by means of 3-day incubation at 30°C in selection plates containing minimal media minus histidine (His) and/or tryptophan (Trp). These yeast transformants were stored at –80°C for subsequent AR function assays.

Western blot assays

Yeast transformants were grown in selection medium to $OD_{600} \approx 1.0$. The collected cells were washed twice with RIPA buffer and resuspended in an equal volume of RIPA buffer. The yeast suspension was homogenized with 1 volume of acid-washed, sterilized, and chilled glass beads (0.45 mm) by vortexing at maximum speed for 5 minutes in the cold room. Supernatants were collected and then centrifuged for 10 minutes at 10,000 g to remove cell debris. Approximately 50- μ g of protein was separated on a 10% SDS-PAGE mini-gel and transferred to a nitrocellulose membrane. The AR, SRC-1, and TIF2 were detected using specific antibodies (anti-AR, PG-21 from Upstate; anti-SRC-1 and anti-TIF2 from BD Biosciences).

Coimmunoprecipitation

Yeast transformants were grown overnight in selection medium to $OD_{600} \approx 1.0$ in the presence of a physiological level (10^{-9} M) of DHT that was added to all protein interaction buffers. Supernatants prepared as just described were precleaned with rabbit IgG and PBS-washed protein A-agarose beads. Approximately 100 μ g of precleaned yeast cell lysate was incubated with 3- μ g anti-AR antibody overnight at 4°C on a rocker. Then, 50- μ L protein A-beads in an equal volume of RIPA buffer was used to capture immunocomplexes. The beads were pelleted at 1000 g and washed thrice with 700- μ L RIPA buffer. The washed beads were resuspended in 50- μ L 2 $\times$ Laemmli sample buffer. The samples were then denatured, separated (20 μ L) on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed using specific coactivator antibodies.

Luciferase assays

The transcriptional activities of the AR in yeast or in mammalian cells were assayed using a luciferase reporter gene assay. In yeast, the yARE-Luc reporter strain expressing the AR and/or coactivator was incubated overnight in selection medium supplemented with different concentrations of ligands as indicated and grown to $OD_{600} \approx 1.0$. The yeast cells were pelleted and resuspended in 0.5-mL 1.2 M sorbitol. After treatment with 50- μ L Zymolyase-20T (4 mg/mL; ICN) at 37°C for 30 minutes, the yeast cells were then washed twice using cold PBS and lysed using 1 $\times$ passive lysis buffer (Promega). After centrifugation to remove insoluble materials, the concentration of protein in the yeast lysates was assessed. Luciferase activity was measured by combining 10- μ L cell lysate with 50- μ L luciferin reagent (Promega) and then immediate reading in a luminometer. Data were calibrated as relative light units (RLU) per μ g protein. To achieve consistent and reproducible results, the yeast cultural conditions were strictly adhered to leading to almost identical yeast OD_{600} readings in these assays. In addition, a DHT/AR-induced luciferase reaction was prepared and used as an external quality control in all yeast experiments. In assays of mammalian cells, AR-null PC3 cells were plated in 24-well plates at 5×10^4 cells/well and grown for 24 hours in androgen-depleted medium. Co-transfection of PC3 cells was performed with the AR expression plasmid (100 ng), the pGL3E/probasin-Luc reporter vector (100 ng), and the pRL-SV40 *Renilla* luciferase plasmid (50 ng; Promega) as an internal control, using lipofectin. The next day, the cells were fed with fresh medium with or

without ligands. After 24 hours, luciferase levels were measured using a dual luciferase reporter assay (Promega) in an EG & G Berthold LB96V MicrolumamatPlus microplate luminometer (Perkin Elmer-Wallac, Inc.).

Results

Development of a yeast AR luciferase assay

A yARE-Luc strain was generated that expresses an ARE-CYC1 promoter-driven luciferase gene which is integrated into the yeast chromosomes at the *URA3* locus, as verified by PCR amplification of the luciferase gene from yeast genomic DNA extracts (data not shown). The reporter strain for background luminescence resulting from the spontaneous oxidation of the luciferin reagent by components of the yeast lysates was first evaluated. Vector-only transformants were cultured in selection medium containing 10^{-9} M DHT. Yeast at an $OD_{600} \approx 1.0$ was harvested, and lysates were prepared. The average background luciferase activity from four independent experiments was $2.1 \pm 0.1 \times 10^3$ RLU per μ g protein. Next, a test was conducted to check whether the WT AR was able to transcriptionally activate the reporter in the presence of different ligands. The yARE-Luc yeast was transformed with the WT AR expression plasmid and grown in selection medium containing β -estradiol (E2, 10^{-8} M), BIC (5.0 μ M), or DHT (10^{-10} or 10^{-9} M). After expression of AR protein was verified using Western blot assay, luciferase activity was determined in the yeast lysates. The AR activity was stimulated by DHT in a dose-dependent fashion but was not induced by E2 or BIC (Fig. 1). The sensitivity of the AR luciferase assay was evaluated by analysis of luciferase

FIG. 1. Transactivation of luciferase by the androgen receptor (AR) in yeast. The yARE-Luc yeast strain was transformed with empty vector (V) or the wild-type (WT) AR expression vector and grown in medium containing 5.0 μ M bicalutamide (BIC), 10^{-8} M β -estradiol (E2), vehicle (0), or different concentrations of dihydrotestosterone (DHT). Ligand-induced luciferase was measured in yeast lysates, and relative light unit per μ g protein was calculated. Luciferase activity was expressed as fold increase relative to AR expression vector in the absence of DHT (0), which was set to 1. The assay was repeated four times with each assay being performed in four replicates, and similar results were obtained each time. Results are shown as mean $\pm$ SE ($n=4$). Inset, Western blot for AR protein expression in yeast. vl, vehicle.

activity in serial dilutions of the AR transformants. A linear relationship between enzyme activity and yeast number was identified (data not shown).

Next, this assay was used to test the ability of CaP-derived AR mutants to transactivate the luciferase. The yARE-Luc cells were separately transformed to stably express the WT AR, the PF AR-S759P mutant that exhibits decreased activity in response to a normal DHT level,¹⁴ the loss-of-function (LOF) AR-C619Y mutant that fails to respond to DHT stimulation,^{14,19} or the GOF AR-K580R or GOF AR-T877A mutant that can be promiscuously activated.¹⁴ These AR stably transformed yeast clones were grown in selection medium containing different concentrations of DHT, and their AR-derived luciferase activity was analyzed. As expected, these ARs failed to transactivate luciferase in the absence of DHT, except for the GOF AR-K580R mutant that induced weak luciferase activity (Fig. 2A). This constitutive weak transactivation of unliganded AR-K580R may be due to its binding to activator protein-1 (AP-1)-like binding sites that had been identified in the yeast *CYC1* minimal pro-

motor. The AR-K580 residue is highly conserved, and equivalent mutations occur at K563 of the rat AR as well as at K206 of the estrogen receptor, K72 of the thyroid hormone receptor, and K461 of the rat glucocorticoid receptor, all of which have been found to enhance AP-1 activity.^{20–22} In the presence of a castration level (10^{-10} M) or a physiological level (10^{-9} M) of DHT, the LOF AR-C619Y could not transactivate luciferase, whereas WT AR and two GOF AR mutants were able to transactivate luciferase in a dose-dependent manner. Compared with the WT AR and GOF ARs, PF AR-S759P was moderately activated only in the presence of 10^{-9} M DHT (Fig. 2A). To test whether other ligands activate these ARs in yeast, luciferase activity was determined in the presence of 10^{-6} M DHEA, 10^{-8} M progesterone and 10^{-8} M E2. These nonandrogenic ligands were able to activate the GOF AR mutants to different extents, but they failed to activate the WT, PF, or LOF ARs (Fig. 2B). Additionally, Western blot assay of these yeast clones was performed to determine whether the observed differences in enzyme activity were the result of different protein expression levels of the AR. The

FIG. 2. Yeast transactivational assays of the WT AR and AR mutants. **(A)** Transactivational capacities of the WT AR (WT AR), a partial-function (PF) AR-S759P mutant (PF-S759P), a loss-of-function (LOF) AR-C619Y mutant (LOF-C619Y), and two gain-of-function (GOF) AR mutants (GOF-K580R and GOF-T877A) in the absence or presence of a castrate (10^{-10} M) or a physiological (10^{-9} M) level of DHT. **(B)** The transactivational capacities of the WT AR, the AR-S759P, the AR-C619Y, the AR-K580R, and the AR-T877A in response to 10^{-6} M dehydroepiandrosterone (DHEA), 10^{-8} M progesterone (Pg), and 10^{-8} M β -estradiol (E2). In both **(A)** and **(B)**, the AR activity is expressed as fold-increase relative to that of the unliganded WT AR. The assay was repeated four times with each assay being performed in four replicates, and similar results were obtained each time. Results are given as mean $\pm$ SE ($n = 4$). **(C)** Western blot assay of WT and mutated AR expression in yeast. **(D)** Luciferase assays of the transactivational capacities of the WT AR and the GOF AR-K580R in PC3 prostate cancer (CaP) cells in the presence of 10^{-9} M E2, 10^{-6} M DHEA, or 10^{-9} M DHT. After calibration with the internal control, luciferase activity is expressed as fold increase over the unliganded WT AR. The assay was repeated thrice with each assay being performed in three replicate wells, and similar results were obtained each time. The representative results are shown as mean $\pm$ SD ($n = 3$). The physiological levels of these hormones in males are DHT, 1.03×10^{-9} – 2.93×10^{-9} M; estradiol, 3.7×10^{-11} – 1.84×10^{-10} M; Pg, 4×10^{-10} – 3.1×10^{-9} M, DHEA, ~ 400 nM in prostate tissue. The castration level of DHT is $\sim 10\%$ of the physiological level.⁵⁰

equivalent expression of AR protein was found for the different yeast clones (Fig. 2C).

To determine whether AR activity in yeast is comparable to that in mammalian cells, AR luciferase assay was performed in mammalian cells. The WT AR and GOF AR-K580R mutant were cloned into pcDNA3.1⁺ plasmids (Invitrogen) and were separately cotransfected with the pGL3E/probasin-Luc reporter vector into PC3 CaP cells that lack AR expression. Transfected cells were grown in the presence or absence of DHT, DHEA, or E2. In the absence of ligands, only background signals were detected in the WT AR transfectant and the GOF AR-K580R transfectant. As anticipated, the WT AR transactivated the probasin promoter in the presence of 10^{-9} M DHT, but it failed to do so in the presence of 10^{-6} M DHEA or 10^{-9} M E2 (Fig. 2D). In response to DHT, the AR-K580R mutant activated the probasin promoter. This mutant was also activated to different extents by DHEA and E2 (Fig. 2D). These results show that the findings from the yeast assay agree with those obtained from the mammalian assay. Taken together, these results indicate that the yeast AR luciferase system is a valid model for quantitating AR transactivation.

Short polyQ tract modulates the activity of PF AR mutants

Several epidemiological surveys have suggested that short CAG repeat lengths in exon 1 of the AR gene may be associated with the pathogenesis of CaP.^{11,12,23,24} However, how alterations of CAG repeat length affect the biology of CaP remains to be defined. It was proposed that contracted polyQ tracts encoded from short CAG repeat lengths increase the transactivational capacity of the AR. This has been well documented for the WT AR.^{10,25,26} and recently for the GOF AR-T877A mutant.¹⁵ Whether short CAG repeat lengths affect the transactivation of other CaP-derived AR mutations is not known. The effects of CAG repeat lengths on several

additional AR mutants were, therefore, evaluated. It was first determined whether various polyQ lengths affected the activities of the WT AR, the GOF AR-K580R, and the GOF AR-T877A mutant. These three AR alleles were engineered to contain 17, 25, or 33 CAGs, and they were stably expressed in the yARE-Luc yeast reporter strain. AR-transactivated luciferase activity was measured in the presence of 10^{-10} or 10^{-9} M DHT. Consistent with previous findings in mammalian cells,²⁶ it was observed that an inverse relationship existed between polyQ length and luciferase activity. As shown in Figure 3A, in the presence of 10^{-10} M DHT, the 17-CAG WT AR showed a 40% greater activity than the 25-CAG isoform that had a 24% greater activity than the 33-CAG isoform; in the presence of 10^{-9} M DHT, 17-CAG WT AR induced a 14% greater activity than the 25-CAG isoform and this, in turn, had a 13% greater activity than the 33-CAG isoform (all $p < 0.05$). The 17-CAG repeat length also induced 18% and 31% increases in the activity of GOF AR-K580R and GOF AR-T877A mutants in the presence of 10^{-10} M DHT, respectively, relative to their 25-CAG counterparts ($p < 0.05$). However, the short CAG repeat did not significantly affect the luciferase activity in the presence of 10^{-9} M DHT (Fig. 3A). This phenomenon was also observed for the GOF AR-H874Y mutant that was detected in 22Rv1 CaP cells (data not shown).

It was previously found that 32% of CaP-derived AR mutations resulted in the receptor having PF activity.¹⁴ However, the effect of CAG repeat length on the functionality of these PF AR mutants has not yet been determined. The influence of altered polyQ lengths on activity of PF AR mutants was, therefore, investigated. Since only the short CAG lengths are linked to CaP aggressiveness,²⁷ the 17-CAG repeat length was focused on. Based on a previous study conducted by us,¹⁴ four representative PF mutant alleles were selected (AR-L574P, AR-G683A, AR-S759P, and AR-F891L), all of which were engineered to contain the 17-CAG repeat. It was investigated whether their protein products were able to transactivate

FIG. 3. Evaluation of the effects of polyQ tract lengths on AR transactivation. **(A)** Analysis of the transactivational capacities of the WT AR, the GOF AR-K580R (GOF-K580R), and the GOF AR-T877A (GOF-T877A) having 17, 25, or 33 Glus (17CAG, 25CAG, or 33CAG) in the presence of various DHT concentrations. Luciferase activity is expressed as fold-increase relative to the unliganded WT AR having 25-Glins. **(B)** Analysis of the transactivational capacities of four PF AR mutants (L574P, G683A, S759P, and F891L) and the LOF AR-C619Y (C619Y) in the presence of 10^{-9} M DHT. Luciferase activity is expressed as fold-increase relative to that of individual unliganded PF AR mutants. The assays were repeated four times with each assay being performed in four replicates, and similar results were obtained each time. Results are shown as mean $\pm$ SE ($n = 4$).

luciferase. In the presence of 10^{-9} DHT, it was found that a 43%–105% increase in luciferase activity occurred in the four PF AR mutants with 17-Gln tracts compared to their 25-Gln counterparts (all $p < 0.05$, Fig. 3B). In the presence of 10^{-10} M DHT, these PF AR mutants having 17- or 25-Glns transactivated a slight increase in luciferase activity over the background signal (data not shown). The results shown in Figure 3A and B indicate that short CAG length may modulate the activity of the WT AR and PF AR mutants, and, to a lesser degree, GOF AR mutants.

p160 coactivators enhance the activity of AR

Although progression of CaP cells to castrate resistance is often accompanied by overexpression of SRC-1 and/or TIF2,^{7,28,29} the direct influence of these two p160 coactivators on the activity of different AR mutants has not been well investigated. The quantitative yeast system was, therefore, used to assess modulations of AR activity by SRC-1 and TIF2. Six AR alleles were selected for investigation. These included 25-CAG WT AR and its 17-CAG counterpart, two GOF AR mutants (AR-K580R and AR-T877A), and two PF AR mutants (AR-S759P and AR-F891L). Both GOF and PF AR mutant alleles contained 25 CAGs. yARE-Luc yeast cells were cotransformed with individual AR alleles and SRC-1 or TIF2. Their expression levels and protein interactions were examined. The results shown in Figure 4 demonstrate that DHT-liganded AR proteins were able to associate with SRC-1 or TIF2 in yeast. SRC-1- or TIF2-modulated transactivation of

luciferase was measured in these yeast cotransformants. In the absence of DHT, coexpression of the AR and the coactivator did not significantly enhance background signal in these assays. In the presence of 10^{-9} M DHT, the yeast clones that coexpressed the AR and the coactivator exhibited a considerable elevation of luciferase activity compared with those clones that lack the coactivator, with approximately a 1.0-fold increase in the WT ARs, two-fold increase in the PF AR mutants, and a 1.0–1.5-fold increase in the GOF AR mutants (Fig. 5A, all $p < 0.01$). There were no differences between SRC-1 and TIF2 in their ability to induce AR activity. These data indicate that SRC-1 and TIF2 are able to efficiently modulate the WT AR as well as the PF and GOF AR mutants. Interestingly, coexpression of the 17-CAG WT AR and one coactivator induced an average 20% increase in enzyme activity compared with their 25-CAG counterparts ($p < 0.05$). This suggests that the p160 coactivator and a short polyQ tract act cooperatively to modulate the WT AR activity.

Since BIC antagonizes androgens and inhibits interactions between the WT AR and the p160 coactivators, thereby diminishing AR activity,³⁰ the effect of BIC on inhibition of ARs that have different functionalities was evaluated. As shown in Figure 5A, treatment with 5.0 μ M BIC resulted in dramatic reduction of DHT-induced luciferase activity in yeast that coexpresses a coactivator (SRC-1 or TIF2) and the WT AR or a PF AR mutant (all $p < 0.01$). However, these antagonistic effects were not evident for AR-K580R and AR-T877A. In the absence of a p160 coactivator, BIC antagonized only 18% of DHT-liganded AR-T877A activity ($p > 0.05$),

FIG. 4. Immunoprecipitation (IP) analyses of the AR-SRC-1 interaction (A) and the AR-TIF2 interaction (B). Individual stable yeast clones co-expressing AR and SRC-1 or AR and TIF2 were grown to ~ 1.0 OD₆₀₀ in medium with or without 10^{-9} M DHT. Total soluble yeast protein was isolated. 50 μ g protein was analyzed using Western blotting (WB) to detect the expression of the AR and coactivators (input). 100 μ g protein was immunoprecipitated using anti-AR antibody in the presence of 10^{-9} M DHT. Immunoprecipitated products were diluted in 50 μ L Laemmli sample buffer, and 20 μ L were detected for AR-bound coactivators using specific anti-SRC-1 or anti-TIF2 antibody. AR-bound coactivators were quantitatively assessed by scanning the protein bands of the input and the anti-AR-precipitated coactivator. An average of 8% (7%–16%) input was immunoprecipitated. Rabbit IgG-precipitated products and anti-AR-precipitated unliganded AR complexes were used as negative controls. WT-25CAG, the WT AR having 25 CAGs; WT-17CAG, the WT AR having 17 CAGs; PF-S759P, the PF AR-S759P mutant; PF-F891L, the PF AR-F891L mutant; GOF-K580R, the gain-of-function AR-K580R mutant; GOF-T877A, the gain-of-function AR-T877A mutant.

FIG. 5. (A) Evaluation of the effects of SRC-1 and TIF2 coactivators on transactivation of the WT AR (WT-25CAG and WT-17CAG) and mutated ARs (PF-S579P, PF-F891L, GOF-K580R, and GOF-T877A) in the presence of 10^{-9} M DHT or 10^{-9} M DHT plus 5 μ M BIC. The transactivational activity of each AR is expressed as a fold-increase over the luciferase activity measured for the corresponding unliganded AR. The assay was repeated thrice with each assay being performed in four replicate tubes, and similar results were obtained each time. Results are shown as mean $\pm$ SE ($n = 3$). (B) IP analyses of the AR-SRC-1 interaction (left) and the AR-TIF2 interaction (right). Individual yeast clones coexpressing AR and SRC-1 or AR and TIF2 were grown to ~ 1.0 OD₆₀₀ in the presence of 5.0 μ M BIC. Total soluble yeast protein was isolated. 50 μ g protein was used to detect the expression of AR and individual coactivators, and 100 μ g protein were analyzed for their interaction. Of six ARs tested, only GOF AR-K580R and GOF AR-T877A can bind coactivators. No signal was detected in rabbit IgG-precipitated products and in anti-AR-precipitated products in the absence of BIC (data not shown).

whereas it did not affect AR-K580R activity. Notably, BIC failed to antagonize the two GOF AR mutants in the presence of DHT and coactivator (Fig. 5A). To investigate why this occurred, the interactions between the GOF AR mutants and the p160 coactivators were examined. Similar to a previous finding that BIC-liganded AR-T877A interacted with the ARA70 coactivator,³¹ it was observed that BIC promoted the association of GOF AR mutants with p160 coactivators (Fig. 5B). Further, luciferase activity in BIC-treated yeast clones that coexpress the GOF AR mutants and coactivators (data not shown) was detected. These results indicate that BIC weakly affects the coactivator-induced activity of GOF AR mutant. Although BIC has been shown to be a pure AR antagonist and used for treatment of patients with CaP, studies have shown that the compound is a mixed agonist-antagonist agent,^{32,33} and it can stimulate AR binding to

DNA³⁴ and partially activate the WT AR transfected into DU145 cells³² and some mutated ARs such as AR-W741C³⁵ and AR-V730M,³² as well as AR-T877A in androgen-independent LNCaP sublines.^{33,36} Further, Comuzzi found that BIC did not block R1881 androgen-induced luciferase activity in DU145 cells transfected with the AR-V730M and the CBP coactivator.³² Taking Comuzzi's finding together, our results indicate that in the presence of BIC and DHT, the relative weak agonistic effect of BIC may be dominated by the stronger agonistic effect of DHT.

Combined influence of short polyQ tract and TIF2 on AR transactivation

Since PF AR mutations occur in one third of CaP-derived AR mutants,¹⁴ whether a combination of a short polyQ tract

and a p160 coactivator cooperatively affect the transactivation of PF AR mutants was investigated. To this end, yARE-Luc reporter yeast cells were cotransformed with *TIF2* and a PF AR mutant (*AR-S759P* or *AR-F891L*) having a 17- or 25-CAG repeat. In the presence of 10^{-9} M DHT and TIF2, 17-CAG *AR-S759P* and 17-CAG *AR-F891L* generated 40% and 58% increases in the luciferase activity, respectively, over 25-CAG *AR-S759P* and 25-CAG *AR-F891L* (Fig. 6A, $p < 0.05$). Since androgen ablation therapy results in reduction of androgen levels in CR CaPs,⁷ it was also determined whether the 17-Gln tract and TIF2 cooperatively modulated AR activity in the presence of a low level (10^{-10} M) of DHT. Although luciferase activity (RLU/ μ g protein) induced by 10^{-10} M DHT was lower than that induced by 10^{-9} M DHT, the 17-Gln tract in combination with TIF2 stimulated 65% and 84% increases in transactivation of *AR-S759P* and *AR-F891L*, respectively, compared with the 25-Gln and TIF2 cotransformants (Fig. 6B). In these experiments, this modulation was not observed for the LOF *AR-C619Y*. This is consistent with the previous finding that R1881-liganded *AR-C619Y* bound with SRC-1, resulting in formation of transcriptionally inactive cytoplasmic aggregates.¹⁹

Activation of mutant ARs by DHEA is a potential mechanism for achieving castrate resistance.⁷ It was previously

FIG. 6. Evaluation of the complex effects of a 17-Gln tract and the TIF2 coactivator on the transactivation of PF *AR-S759P* (PF-S759P) and PF *AR-F891L* (PF-F891L) in the presence of 10^{-9} M (A) or 10^{-10} M (B) DHT. LOF *AR-C619Y* (LOF-C619Y) was used as the negative control. All results are expressed as fold increase compared with individual unliganded AR mutants having a 25-Gln tract. The assays were repeated thrice with each assay being performed in four replicate tubes, and similar results were obtained each time. Results are shown as mean $\pm$ SE ($n = 3$).

FIG. 7. Evaluation of the complex effects of a 17-Gln tract and the TIF2 coactivator on the transactivational capacities of the WT AR, the PF *AR-S759P* (PF-S759P), and the GOF *AR-T877A* (GOF-T877A) in the presence of 10^{-6} M DHEA. All results are expressed as fold increase over individual unliganded ARs having a 25-Gln tract. The assays were repeated thrice with each assay being performed in four replicate tubes, and similar results were obtained each time. Results are shown as mean $\pm$ SE ($n = 3$).

observed that 45% of CaP-derived AR mutants could be activated to different extents by DHEA.¹⁴ It is interesting to know whether short CAG repeat lengths and AR coactivators modulate the transactivational activity of these AR mutants in the presence of DHEA. To address this issue, the complex effects of TIF2 and a 17-Gln tract on the transactivational capacities of three ARs (WT AR, *AR-S759P*, and *AR-T877A*) in the presence of a physiological level (10^{-6} M) of DHEA were evaluated. When compared with transformants that coexpressed TIF2 and a 25-Gln AR, the combination of TIF2 and a 17-Gln AR increased luciferase activity by 67% and 87% in the WT AR and the PF *AR-S759P*, respectively (Fig. 7, $p < 0.01$). However, the increase was not observed in the GOF *AR-T877A* mutant although DHEA highly activated this mutant in the presence of TIF2 (Fig. 7). These data suggest that short polyQ tracts, in combination with coactivators, are able to modulate the function of the WT AR and PF AR mutants in response to DHEA.

Discussion

A yeast system to quantitatively assess the transactivational capacity of the AR has been developed. Our results show that this assay is rapid, sensitive, and reproducible. Moreover, the AR activities found in the yeast system are usually closely similar to those found in mammalian cells. In this article, the influence of three different kinds of modulators of AR transactivation, p160 coactivators, shorter CAG repeat length, and AR mutations were evaluated. Since yeast lacks homologs to the AR or its coactivators, using the yeast model avoids such confounding factors. It was found that a combination of a short CAG repeat and the presence of a p160 coactivator enhances transactivation of the AR for both the WT AR and PF AR mutants.

In advanced CaP, one mechanism that enhances AR activity is upregulation of coactivators.⁷ A large number of putative AR coactivators have been identified in CaP. Although these coactivators lack DNA binding domains and, therefore, are unable to directly transactivate gene expression, they are able to interact with liganded AR and thereby

increase the stability of the receptor.³⁷ This results in an increased transactivational capacity for the AR. In this study, two p160 coactivators, SRC-1 and TIF2, were examined for their influence on AR activity, and it was found that they significantly enhanced transactivation of the WT AR and PF and GOF AR mutants. It is of particular interest that the presence of these p160 coactivators upregulates the transactivational capacity of PF AR mutants in response to a castration level of androgen and also to the adrenal androgen, DHEA. This provides a mechanistic explanation for how CaPs expressing PF AR mutants can become able to grow in a castrate environment. The transactivational activity induced by p160 coactivators is less in yeast than in mammalian cells.^{7,38,39} This can be partly attributed to the lack of some mammalian regulating factors in yeast that may directly affect AR activity or may enhance the interaction of the AR with its coactivators. However, as just noted, the absence of mammalian regulating factors in yeast permits us to measure the pure effects of individual modulators and allows us to assess their combined effects on AR activity.

Previous studies have found an inverse relationship between AR transactivation and CAG repeat length.^{26,40} On average, there is a 1.7% increase in AR activity for each decrease in the number of CAG repeats.¹⁰ Consistent with previous findings, using the yeast model, it was observed that the WT AR with a short polyQ tract exhibits increased transactivation in the presence of a physiological level of DHT. In addition to this effect on WT AR, it was found that a 17-Gln tract enhanced transactivation of several CaP-derived PF AR mutants. Although the extent of these increases of AR activity is less than those induced by p160 coactivators, it is possible that the long-term cumulative effect of such short CAG repeats on AR functionality may speed up CaP progression. Although the molecular mechanism underlying the effects of a short CAG repeat length on AR activity remains to be defined, a recent study suggests that a shorter polyQ tract in the AR can reduce its N-terminal and C-terminal interactions. This would result in increased transactivation due to decreased inhibition of the Activation Function 1 (AF1) domain of exon 1 of the AR.¹⁰ It is also possible that a short polyQ tract that is positioned between the ²³FQNLF²⁷ motif and the AF1 may increase secondary structural order in the N-terminal activation domain of the AR. This could increase the interaction of the ²³FQNLF²⁷ motif with the AR ligand-binding domain, resulting in more effective recruitment of coactivators and components of the transcription machinery.¹⁰

The combined action of a short polyQ tract and p160 coactivators on the transactivation of WT AR and of several PF AR mutants was assessed. The combination of a 17-Gln tract and a coactivator significantly increased luciferase activity compared with the presence of a 25-Gln tract and the coactivator, although the short polyQ tract caused less enhancement of AR activity than that induced by the coactivator. It is evident, however, that both the shorter polyQ tract and the coactivators act to cooperatively modulate AR activity. It was further found that the combination of a short polyQ tract and the expression of a p160 coactivator significantly increased the response of the WT AR and PF AR mutants to the adrenal androgen, DHEA, the most abundant steroid molecule found in humans. Although androgen ablation treatment causes more than a 90% reduction in plasma

androgen levels, it induces only a slight decrease in plasma DHEA levels.⁴¹ This adrenal steroid has been reported to contribute to the progression of CaP cells that expresses a GOF AR mutant such as the T877A-AR mutant of LNCaP cells and the H874Y-AR mutant of 22Rv1 cells.^{7,42} Whether DHEA stimulates the transactivation of WT AR or PF AR mutants has not previously been investigated. Our findings indicate that in the presence of a short polyQ tract and overexpression of a p160 coactivator, DHEA stimulates the activity of both WT and PF AR mutants. It is, therefore, possible that this combination of factors might promote the progression of CaP.

In the DHEA metabolic pathway of human cells, 17 β -hydroxysteroid dehydrogenase (17 β -HSD) catalyzes the conversion of DHEA into androstenediol (adiol), which is subsequently converted by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) into testosterone.⁴³ Due to the lack of expression of 17 β -HSD and 3 β -HSD in yeast,⁴⁴ our finding that DHEA increases luciferase activation raises the question of how this adrenal steroid activates the AR in yeast. Published data provide a clear answer to this question. Vico reported that *S. cerevisiae* expresses several polypeptides similar to human 17 β -HSD. Of these, the AYR1 polypeptide efficiently catalyzes the conversion of DHEA into adiol in yeast.⁴⁵ Adiol has been shown to strongly activate the AR.⁴⁴ In addition, DHEA can directly bind with the AR. Although the affinity of the AR to DHEA is less than that to testosterone or DHT, this adrenal steroid exerts a positive effect on AR activity.⁴⁶ Therefore, DHEA-induced transactivation of luciferase by the AR in yeast may result from its metabolic conversion into adiol and, to a lesser degree, from direct binding to the AR.

The finding that overexpression of a p160 coactivator, shorter CAG repeat, and AR mutations can cooperatively modulate AR activity could be of considerable importance in the initiation and progression of CaP. Even in a CaP tumor that expresses WT AR which has a short CAG repeat length, the cooperative modulation of AR activity by a p160 coactivator and the short CAG length might affect early events in carcinogenesis. Although the frequency of the combination of these factors in early CaP has yet to be determined, these three modulators are known to be common in the late stage of CaPs. A number of studies have shown that AR mutations occur frequently in advanced CaPs^{47,48}; that short CAG repeat lengths are relatively frequent in CaPs after androgen ablation treatment^{10,49}; and that androgen ablation selects for CaP cells expressing increased SRC-1 and/or TIF2.^{28,29} Thus, their cooperative modulation of AR activity could well play a key role in promoting progression of CaP to castrate resistance, particularly in CaPs that express PF AR mutations. It is, therefore, possible that modulated AR activity might serve as a biomarker for predicting the progression of CaP and suggests that their presence could provide a valuable objective guide to response of individual patients to AAT. In combination with other techniques such as PCR-DNA sequencing for detection of AR mutations and identification of short CAG repeat lengths, as well as the immunochemical detection of coactivator expression, the AR luciferase yeast assay might be useful for quickly and efficiently quantitating the extent of activation of AR transactivation in individual clinical CaP samples. Although we anticipate that finding that AR activity is significantly modulated by these factors and that this will be correlated with clinical outcome, much

work needs to be performed before the clinical utility of data from the yeast quantitative assay can be determined.

In summary, using the AR luciferase yeast assay, it has been observed that the cooperative enhancement of AR activity is caused by p160 coactivators, short polyQ length, and/or AR mutations. Our study not only provides information about this cooperative modulation of AR activity, but it also suggests potential clinical applications of the yeast system. Our findings suggest the possibility that these AR modulators may be involved early in carcinogenesis and predict that CaP cells expressing an AR in the presence of multiple AR modulators may be more likely to progress to a CR state.

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Disclosure Statement

The authors declare that there are no financial conflicts of interest.

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