Androgen Receptor Requires JunD as a Coactivator to Switch on an Oxidative Stress Generation Pathway in Prostate Cancer Cells

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Abstract

Relatively high oxidative stress levels in the prostate are postulated to be a major factor for prostate carcinogenesis and prostate cancer (CaP) progression. We focused on elucidating metabolic pathways of oxidative stress generation in CaP cells. Previously, we showed that the transcription factor JunD is essential for androgen-induced reactive oxygen species (ROS) production in androgen-dependent human CaP cells. We also recently showed that androgen induces the first and regulatory enzyme spermidine/spermine N1-acetyltransferase (SSAT) in a polyamine catabolic pathway that produces copious amounts of metabolic ROS. Here, we present coimmunoprecipitation and \textit{Gaussia} luciferase reconstitution assay data that show that JunD forms a complex with androgen-activated androgen receptor (AR) \textit{in situ}. Our chromatin immunoprecipitation assay data show that JunD binds directly to a specific SSAT promoter sequence only in androgen-treated LNCaP cells. Using a vector containing a luciferase reporter gene connected to the SSAT promoter and a JunD-silenced LNCaP cell line, we show that JunD is essential for androgen-induced SSAT gene expression. The elucidation of JunD-AR complex inducing SSAT expression leading to polyamine oxidation establishes the mechanistic basis of androgen-induced ROS production in CaP cells and opens up a new prostate-specific target for CaP chemopreventive/chemotherapeutic drug development.

Introduction

Approximately 1% to 5% of the oxygen that we breathe in is converted to reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), superoxide, and hydroxyl radical (reviewed in ref. 1). When cellular ROS production exceeds detoxification capacity, oxidative stress occurs (2, 3). Damage to cellular proteins, DNA, RNA, and phospholipids due to oxidative stress (4–9) as well as ROS molecules acting as signals for promoting cell proliferation (10) are believed to contribute to cancer development. The strongest evidence thus far is ROS-induced oxidative damage products in human and transgenic mouse prostatectomy specimens. Both mouse and human prostate tumor cells are reported to have higher ROS-induced macromolecular damages compared with normal prostatic epithelium (11, 12). Accumulating evidence shows that ROS play a key role in occurrence and recurrence of prostate cancer (CaP) as well as its progression from androgen dependence to androgen independence (11–15).

Androgen signaling is one source of ROS generation in prostatic epithelial cells (16, 17). Androgen binding to androgen receptor (AR) initiates a cascade of events leading to ROS generation in prostate cells (17–19). We established that one pathway of androgen-induced oxidative stress involves activation of activator protein-1 (AP-1) transcription factor JunD (19, 20), followed by induction of enzyme spermidine/spermine N1-acetyltransferase (SSAT) that initiates a major polyamine oxidation pathway (21). As prostatic epithelia produce a large excess of polyamines, induction of polyamine oxidation could result in high ROS levels in the prostate. We have shown that inhibiting androgen-induced ROS production using different small-molecule inhibitors of the androgen signaling pathway or polyamine oxidation inhibits cell growth and androgen-induced ROS generation in cultured human CaP cells as well as tumor growth in transgenic adenocarcinoma in the mouse prostate model (21, 22).\textsuperscript{4}

Because the SSAT gene promoter sequence contains no AR-binding element (ARE), the exact mechanism of how AR may induce SSAT gene expression remains unknown. To develop more potent CaP chemopreventive agents that can specifically block this pathway, we focused on elucidating the mechanism of androgen-induced SSAT gene expression.

We previously showed that androgen activation of AR in LNCaP human CaP cells induces AP-1 transcription factors Fra-2 and JunD (19). However, only JunD levels and its functional activity remained elevated for 96 hours after androgen treatment when androgen-induced oxidative stress was...
observed (17–20), JunD may either inhibit (23–25) or help (26) cellular ROS production depending on cell type, presence of ROS-generating proteins, growth conditions, etc. Because androgen-induced ROS generation was abrogated by either blocking androgen-induced JunD overexpression with anti-androgen bicalutamide or silencing JunD protein expression using siRNA (19, 20), we concluded that JunD activity is necessary for androgen-induced oxidative stress in LNCaP cells.

Here, we present data clearly showing that (a) JunD is required for androgen-induced SSAT gene expression, (b) activated AR interacts with JunD in situ, and (c) JunD binds directly to the SSAT promoter sequence only in androgen-treated human CaP cells. Based on these results, we hypothesize that AR and JunD form a complex that binds to the SSAT promoter, resulting in SSAT gene expression and consequent high levels of ROS in androgen-treated prostate cells. Induction of SSAT and polyamine oxidation as a main source of ROS production in prostatic epithelia was first reported from our laboratory (27) and further confirmed in our subsequent publication (21). Although other studies implicating the effect of AR-induced CaP cell growth stimulation via ROS production through changes in mitochondrial function and gene expression were published within the last couple of years (28, 29), none of those publications probed deep into the actual biochemical pathway(s) of ROS production in CaP cells. To the best of our knowledge, this is the first demonstration of a possible molecular mechanism of androgen-induced activation of an enzymatic pathway that can be directly related to ROS generation in prostate cells. A clear understanding of this mechanism may open a new avenue of research in the field of therapy and/or prevention of CaP occurrence and progression.

**Materials and Methods**

**Antibodies**

Primary antibodies were as follows: monoclonal antibody against AR (Santa Cruz Biotechnology), polyclonal antibody against JunD (Santa Cruz Biotechnology), polyclonal antibody against Gaussia luciferase (Nanolight Technology), and monoclonal antibody against β-actin (Sigma). Secondary antibodies for immunohistochemistry were as follows: Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen).

**Cell culture**

Androgen-sensitive LNCaP human prostate carcinoma cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; F10 medium) as described before (17). Hep3B human hepatoma cells were obtained from the Small Molecule Screening Facility at the University of Wisconsin Carbone Cancer Center and maintained in RPMI 1640 supplemented with 10% FBS and antibiotics. Cell lines are tested annually for Mycoplasma.

Culture conditions for LNCaP androgen response studies included use of cells passage 40 to 90, hormone-depleted media containing 4% charcoal-stripped FBS plus 1% non-stripped FBS (F1C4), and synthetic androgen R1881 (methyltrienolone; NEN) at 1 nmol/L for maximal induction of JunD and ROS as described before (17, 19, 20). For AR-JunD interaction studies in AR-transfected Hep3B cells, R1881 was used at 2 nmol/L in DMEM medium to maximally induce AR (data not shown).

**Vector construction**

cDNA for human AR was obtained from Open Biosystems. The whole human junD gene (20) was subcloned in a pcI-based vector (Promega). Two sections of the humanized Gaussia luciferase gene, NH2-terminal hGluc1 and COOH-terminal hGluc2, in two separate vectors (30) were kind gifts from Prof. Stephen Michnick (University of Montreal, Montreal, Quebec, Canada). hGluc1 was cloned in frame with the NH2-terminal end of AR in a pcDNA3.1-based vector (Invitrogen) to create vector Gluc1-AR.

The pCl-junD vector was used to fuse hGluc2 in frame at the end of the junD gene after removing the junD stop codon to construct vector JunD-Gluc2. The authenticity of each construct was verified by using BigDye terminator and sequencing via the Biotechnology Center of University of Wisconsin-Madison. The in-frame fusion of each construct was also verified by transfecting each into AR-negative Hep3B cells and analyzing cell lysate by Western blot with AR antibody for Gluc1-AR or antibody for Gaussia luciferase at the COOH-terminal end of the fusion protein for JunD-Gluc2. β-Actin was used to control for protein loading in all Western blot analyses.

**Transfection of constructs into Hep3B cells**

Hep3B cells (5 × 105) were seeded and then, 1 day later, cotransfected with 3 μg each of Gluc1-AR and JunD-Gluc2 constructs or transfected with Gluc1-AR or JunD-Gluc2 alone as negative controls using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer-supplied protocol. Two to 3 hours after transfection, cells were washed and refed DMEM without serum and treated with 2 nmol/L R1881 for 48 hours before collecting cell lysates. Corresponding untreated cells were used as negative controls.

**Bioluminescence activity of Gaussia luciferase in lysates from Hep3B cells transfected with Gluc1-AR and JunD-Gluc2**

Gaussia luciferase activity was measured in 25 μL of Hep3B cell lysates from R1881-treated or untreated control cells using a Gaussia luciferase assay kit from New England Biolabs following the manufacturer-supplied protocol. Biluminoscence activity of the lysate-substrate mixture was read on a single-tube Monolight 2010 luminometer (Analytical Luminescence Laboratory) at 480 nm.

**Immunocytochemistry**

LNCaP cells were grown in F1C4 medium on coverslips for 2 days followed by 3 days of treatment with 1 nmol/L R1881. Immunofluorescent staining was carried out following a published procedure (31) using primary-secondary antibody pairs JunD–Alexa Fluor 594 or AR–Alexa Fluor 488.
Immunoprecipitation

For whole-cell lysates, LNCaP cells were lysed using modified radioimmunoprecipitation assay buffer containing complete protease inhibitors (Roche Applied Sciences). Nuclear and cytoplasmic fractions were prepared and checked for purity using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific, Pierce Biotechnology) following manufacturer-supplied protocol. For immunoprecipitation, lysates were precleared by incubation with 50% protein A–agarose slurry (Pierce). AR antibody (6 μg) and 500 μL precleared lysate (500 μg total protein) were mixed and rocked overnight at 4°C. The immunocomplex was captured with 100 μL of 50% protein A–agarose slurry and then analyzed by Western blotting using JunD antibody. The same immunoprecipitation procedure was repeated for capturing the immunocomplex using JunD antibody and Western blotting using AR antibody. Proper controls with IgG and protein A–agarose were run in parallel.

Transcriptional activity of full-length SSAT promoter in siJunD and vector control cell lines

The full-length SSAT promoter sequence, kindly provided by Dr. Robert Casero (Johns Hopkins University, Baltimore, MD), was amplified and cloned into pGL4-basic vector (Promega) with a firefly luciferase reporter gene. This vector, pGL4-SSAT-luc, was transiently transfected into our published (20) JunD-silenced (siJunD) and vector control LNCaP cell lines. Briefly, 5 × 10^5 siJunD or vector control LNCaP cells were seeded and then transfected 1 day later with 1 μg pGL4-SSAT-luc DNA construct using Lipofectamine 2000. After transfection, cells were treated with 1 nmol/L R1881 or left untreated for 72 hours and then lysed. Luciferase activity was measured in cell lysates by a luciferase assay system kit (Promega) following the manufacturer-supplied protocol.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using a commercially available ChIP assay kit (Millipore), essentially following the manufacturer-supplied protocol. Briefly, 2 × 10^6 LNCaP cells were treated with 1 nmol/L R1881 for 24 hours, protein-DNA was cross-linked by addition of formaldehyde (1% final concentration), cells were lysed, and lysates were sonicated for twenty 10-second pulses with 30-second intervals to shear the chromatin into ~500-bp fragments. Cross-linked protein-DNA was separated into four parts and immunoprecipitated with either 6 μg JunD antibody, 6 μg AR antibody, nonspecific rabbit IgG, or no antibody. Chromatin-antibody complexes were isolated by incubation with 50% salmon sperm DNA/protein agarose slurry. Pelleted agarose was eluted and DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation. Two microliters of this DNA were used for each PCR to determine the presence of SSAT promoter fragment bound to either JunD or AR in the immunoprecipitates. Primers were designed based on the SSAT gene promoter sequence to cover the complete SSAT promoter sequence (GenBank accession number 1103903) as follows: F1,
5′-ggagggctgaagcaggagaatc; R1, 5′-ctcactctattgcccaggctggag; F2, 5′-gagatggcgccattgcactcc; R2, 5′-gagtgcaatggcgccatctcg; F3, 5′-tctgagggtctcccggatcacac; and R5, 5′-acctcggcgagtgacggatagg.

PCR products were run on a 1% agarose gel, purified, cloned into pCR2.1 TOPO vector (Invitrogen), and transformed into TOP10F′-competent cells (Invitrogen). Ten colonies were selected and their plasmids were extracted and sequenced using M13 primer (5′-caggaaacagctatgac).

Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) analysis of SSAT mRNA levels was performed as previously described (21).

Results

AR and JunD coimmunoprecipitate from LNCaP cell lysates

The coimmunoprecipitation of AR and JunD was first shown in whole-cell lysates from LNCaP cells grown under normal F10 medium conditions (Fig. 1A and B).

The immunoprecipitation of AR by rabbit polyclonal antibody against JunD (IP:JunD) was visualized by Western blot analysis using monoclonal antibody against human AR (WB:AR) as shown in Fig. 1A. The immunoprecipitation of JunD by monoclonal antibody against AR (IP:AR) was visualized by Western blotting using antibody against JunD (WB:JunD) as shown in Fig. 1B.

To specifically investigate the effect of androgen, LNCaP cell lysates were prepared after incubation with 1 nmol/L R1881 for 72 hours and analyzed for coimmunoprecipitation of AR and JunD in nuclear and cytoplasmic fractions of untreated versus androgen-treated LNCaP cells are shown in Fig. 1C and D. Immunoprecipitate using JunD antibody that was probed in a Western blot using AR antibody (Fig. 1C) showed that AR-JunD immunocomplex in the nuclear fraction was increased by ∼3-fold (normalized to β-actin) in 1 nmol/L R1881–treated cells compared with low androgen-untreated cells growing in F1C4. Only a small increase was observed in nuclear fractions by IP:AR and WB:JunD (Fig. 1D). This may be due to a difference between the nature of interaction between JunD with its antibody compared with that between AR and its antibody. No difference in AR-JunD immunocomplex was observed in cytoplasmic fractions of R1881-treated versus R1881-untreated cells.

Androgen induces nuclear translocation of JunD in LNCaP cells

To further investigate the effect of androgen treatment on JunD activity in LNCaP cells, immunofluorescence staining was performed to determine JunD localization in untreated versus androgen (1 nmol/L R1881)–treated LNCaP cells. Representative pictures for each condition are shown in Fig. 2. In untreated LNCaP cells, JunD is mostly dispersed in the cytoplasm with negative staining for the nuclei (Fig. 2A). After R1881 treatment, a substantial amount of JunD translocates into the nucleus as shown in Fig. 2B. Using AR antibody and its related fluorescence-tagged secondary antibody, the translocation of AR into the nucleus in R1881-treated LNCaP cells also was observed under the same condition (data not shown), which is consistent with observation reported elsewhere (31). These data suggest that androgen induces simultaneous translocation of JunD and AR into cell nuclei.

Expression of Gluc1-AR and JunD-Gluc2 in Hep3B cells

Because Hep3B cells do not have endogenous AR, this cell line was chosen as a model for AR and JunD interaction studies using a protein complementation assay developed by Remy and Michnick (30). This technique is based on reconstitution of the reporter enzyme Gaussia luciferase in live cells. The gene coding for the enzyme was split into
two sections: NH2-terminal section (Gluc1) and COOH-terminal section (Gluc2). Gluc1 and Gluc2 sequences were separately fused to the NH2 terminus of AR (Gluc1-AR) and the COOH terminus of JunD (JunD-Gluc2), respectively, as shown schematically in Fig. 3A.

To verify in-frame fusion of Gluc1-AR and JunD-Gluc2, cell lysates from Hep3B cells transfected with Gluc1-AR or JunD-Gluc2 were analyzed by Western blotting. Figure 3B shows Western blot analysis using monoclonal antibody against AR. Hep3B cells alone (Fig. 3B, lane 1) or Hep3B cells transfected with control vector (pcDNA3.1; lane 2), control untransfected Hep3B cells; lane 3, Hep3B cells transfected with Gluc1-AR. C, representative Western blot of Hep3B cell lysates analyzed using Gaussia luciferase antibody. Lane 1, Hep3B cells transfected with control vector (pCI); lane 2, control untransfected Hep3B cells; lane 3, Hep3B cells transfected with JunD-Gluc2. B and C, membranes were stripped and probed with monoclonal antibody against β-actin to control for protein loading. Positions of molecular size markers 148 kDa (B) and 98 kDa (C) are shown on the right. Cell lysates were obtained and analyzed by Western blot from six independent transfection experiments for each construct with similar results. D, Gaussia luciferase activity in cotransfected cells: Hep3B cells were cotransfected with Gluc1-AR and JunD-Gluc2 and then treated with androgen (2 nmol/L R1881; gray column) or left untreated (Control; black column). Cell lysates were collected after 48 h, and bioluminescence activity of Gaussia luciferase was assayed by measuring light emitted from reconstituted Gluc1-Gluc2 at 480 nm. Reconstitution of Gluc1-Gluc2 and resulting Gaussia luciferase activity was significantly increased >5-fold in androgen-treated cells compared with untreated control cells. Lysates used for these studies were collected from six independent transfections each run in triplicate. *, P < 10⁻⁸.

**Figure 3.** Western blot and bioluminescence analysis showing expression of Gluc1-AR and JunD-Gluc2 and reconstitution of Gaussia luciferase activity following androgen stimulation in transfected Hep3B cells. A, schematic diagrams for Gluc1-AR and JunD-Gluc2 fusion constructs. B, representative Western blot of Hep3B cell lysates analyzed using AR antibody. Lane 1, Hep3B cells transfected with control vector (pcDNA3.1); lane 2, control untransfected Hep3B cells; lane 3, Hep3B cells transfected with Gluc1-AR. C, representative Western blot of Hep3B cell lysates analyzed using Gaussia luciferase antibody. Lane 1, Hep3B cells transfected with control vector (pCI); lane 2, control untransfected Hep3B cells; lane 3, Hep3B cells transfected with JunD-Gluc2. B and C, membranes were stripped and probed with monoclonal antibody against β-actin to control for protein loading. Positions of molecular size markers 148 kDa (B) and 98 kDa (C) are shown on the right. Cell lysates were obtained and analyzed by Western blot from six independent transfection experiments for each construct with similar results. D, Gaussia luciferase activity in cotransfected cells: Hep3B cells were cotransfected with Gluc1-AR and JunD-Gluc2 and then treated with androgen (2 nmol/L R1881; gray column) or left untreated (Control; black column). Cell lysates were collected after 48 h, and bioluminescence activity of Gaussia luciferase was assayed by measuring light emitted from reconstituted Gluc1-Gluc2 at 480 nm. Reconstitution of Gluc1-Gluc2 and resulting Gaussia luciferase activity was significantly increased >5-fold in androgen-treated cells compared with untreated control cells. Lysates used for these studies were collected from six independent transfections each run in triplicate. *, P < 10⁻⁸.

**Bioluminescence activity of reconstituted Gaussia luciferase in Hep3B cells cotransfected with Gluc1-AR and JunD-Gluc2 is markedly enhanced by androgen treatment**

Cell lysates from Hep3B cells that were cotransfected with both Gluc1-AR and JunD-Gluc2 with or without treatment with androgen (R1881) were collected 48 hours after transfection and analyzed for Gaussia luciferase bioluminescence activity. Results are shown in Fig. 3D. Lysates from cotransfected cells that were not treated with androgen (control) showed very low Gaussia luciferase activity. Lysates from cotransfected cells that were treated with 2 nmol/L R1881 (+R1881) showed significantly higher Gaussia luciferase activity.
activity ( >5-fold; \( P < 10^{-8} \)) than the untreated cotransfected cells. Cells transfected with either of the fusion constructs Gluc1-AR and JunD-Gluc2 individually did not show any measurable Gaussia luciferase activity (data not shown), confirming that the enzyme activity is only observed after both fragments Gluc1 and Gluc2 associate with each other (30). Minor baseline reporter enzyme activity in cell lysates from androgen-untreated cotransfected cells (control) might be due to interaction of residual activated AR remaining after the transfection process, which was performed in medium containing serum that was not stripped of androgen. These data clearly establish an interaction of JunD and androgen-activated AR \( \text{in situ} \) that brings their corresponding fusion proteins Gluc1 and Gluc2 together to reconstitute Gaussia luciferase activity.

**Figure 4.** Androgen-induced increase in SSAT promoter activity is abrogated when JunD is silenced in LNCaP cells. A, LNCaP cells in which JunD is silenced (siJunD) and respective vector control LNCaP cells were transiently transfected with an SSAT promoter luciferase reporter vector and then treated with androgen (1 nmol/L R1881; gray columns) or left untreated (Control; black columns). Cell lysates were collected after 72 h, and firefly luciferase activity was measured. Columns, mean from 18 data points of measured relative light units (RLU) normalized to protein concentration from six independent repeat transfection/treatment experiments (\( n = 3 \) samples per condition for each experiment); bars, SD. \( P \) values were calculated using a two-tailed Student’s \( t \) test assuming unequal variance. B, SSAT mRNA levels as determined by qRT-PCR showing a >25-fold increase in SSAT mRNA in vector control cells and only 10-fold increase in SSAT mRNA in JunD-silenced cells (siJunD). Results are presented as a ratio of mRNA in androgen-treated/androgen-untreated cells after normalizing for corresponding 18S rRNA. Data are mean of three independent observations.

**Activated AR requires JunD to induce transcriptional activity of the SSAT promoter**

The effect of AR activation by androgen on transcriptional activity of full-length SSAT promoter and the necessity of JunD for such effect was studied in LNCaP cells stably transfected with either junD-silenced (siJunD) or control vector that were then transiently transfected with a full-length SSAT promoter–firefly luciferase reporter vector (FLSSAT-luc) followed by androgen treatment (Fig. 4A and B). Androgen treatment (1 nmol/L R1881) caused a significant >16-fold increase in SSAT promoter activity in vector control LNCaP cells compared with corresponding untreated cells (control) (Fig. 4A; \( P = 0.03 \)). Although androgen also caused a small increase in SSAT promoter activity in siJunD cells, where JunD expression is 70% suppressed, the extent of induction was not statistically significant (Fig. 4A; \( P = 0.16 \)). SSAT mRNA levels in LNCaP cells determined by qRT-PCR assay are shown in Fig. 4B. There is an androgen-induced increase in SSAT mRNA (~10-fold) in siJunD cells, but this increase is much less than that observed in vector control cells (~25-fold). Thus, although some increase in SSAT mRNA expression in siJunD cells was observed, the increase is not enough to significantly enhance cellular SSAT protein expression as evident from the insignificant increase in luciferase reporter expression.

**JunD binds to the SSAT promoter \( \text{in situ} \) by ChIP assay**

ChIP assay was performed in LNCaP cells with R1881 treatment to ascertain if activated AR and/or JunD bind to the SSAT promoter site using primer sets targeted to identify the SSAT promoter sequence in the immunoprecipitates (see Materials and Methods). ChIP assay was performed under conditions where only protein-DNA and not protein-protein interactions can be detected (reviewed in ref. 32). Under these conditions, the only PCR product obtained using those primers was from chromatin fragment(s) immunoprecipitated by rabbit polyclonal antibody against JunD using the F1R1 primer pair as shown in Fig. 5A. Using the same F1R1 primer pair, no PCR product was obtained from immunoprecipitates obtained by monoclonal antibody against AR, nor from the nonspecific IgG and no antibody controls. None of the other primer pairs yielded any PCR product from either JunD-, AR-, or IgG-immunoprecipitated chromatin fragments (data not shown).

The sequence data obtained from the PCR product that was cloned in pCR2.1 TOPO indicate the existence of JunD-binding sites at ~574 to ~651 bp upstream in the SSAT promoter sequence that contains multiple half-sites (TGA) of the AP-1 consensus sequence (TGA\(^{GC}GTCA\); Fig. 5B).

**Discussion**

Although it is well established that androgens produce oxidative stress in prostate cells that plays a key role in the occurrence and progression of CaP, the exact molecular mechanism of androgen-induced oxidative stress generation in prostatic epithelia and CaP cells is only recently being elucidated. We previously reported that AP-1 transcription...
factor JunD plays a key role in androgen induction of ROS (20). Recently, we reported that androgen significantly induces the expression and enzymatic activity of SSAT (21), a regulatory enzyme in the polyamine catabolic pathway that produces excess amount of ROS in polyamine-rich prostate cells. In this report, we show a relationship at the molecular level between these two components that establishes the mechanism of androgen-induced ROS generation in prostate cells.

Discovering the mechanism that regulates expression of SSAT is the focus of many studies (33–35). Thus far, binding sites of many important transcription factors in the SSAT gene promoter have been identified (33–35). Because the SSAT gene promoter sequence lacks an ARE, the mechanism of androgen-induced SSAT expression is unclear. Here, we show a direct binding of androgen-activated AR with JunD, and that an induction of SSAT by androgen occurs following an interaction of JunD with a specific sequence in the SSAT promoter only in androgen-treated LNCaP cells, probably due to the formation of an activated AR-JunD complex.

We previously showed that androgen-activated AR induces overexpression of transcription factor JunD as well as activates JunD binding to the AP-1 DNA-binding sequence in LNCaP cells (19). Here, we show that androgen treatment causes AR and JunD to coprecipitate as an immunocomplex from LNCaP cell lysate. Relatively more complex precipitates from the nuclear than from the cytoplasmic fraction (Fig. 1). Androgen treatment induces translocation of JunD into the nucleus in LNCaP cells (Fig. 2) at the same time as AR translocates into the nucleus as shown by immunoprecipitation/Western

**Figure 5.** ChIP assay identifying a binding site for JunD but not AR within the SSAT promoter sequence. ChIP assay studies were carried out in LNCaP cells treated with androgen (1 nmol/L R1881) using primer pairs targeted to identify the SSAT promoter sequence (see text). A, agarose gel electrophoresis of PCR products showing the only PCR product obtained, which was from DNA fragments immunoprecipitated by JunD antibody (JunD lane) using the F1R1 primer pair. Using the same F1R1 primer pair, no PCR product was obtained from immunoprecipitation of chromatin fragments by AR antibody (AR lane) nor from the nonspecific IgG (IgG lane) controls. M, DNA ladder size marker. B, sequence of the PCR product, which was cloned into pCR2.1 TOPO and sequenced using M13 primer, matches −574 to −651 bp of the SSAT gene promoter (National Center for Biotechnology Information accession number 1103903).

**Figure 6.** Schematic diagram showing a possible mechanism of androgen-induced increase in cellular ROS production in CaP cells through an AR-JunD complex. APAO, N′-acetylpolyamine oxidase.
Photoblot of nuclear extract (Fig. 1C) and also reported by other laboratories (31). These observations suggest an interaction of activated AR with JunD in androgen-treated CaP cells that also causes functional activation of JunD.

More direct evidence of AR and JunD interaction was shown using the *Gaussian* luciferase reconstitution assay recently developed to study *in situ* protein-protein interactions (30). The significant reconstitution of *Gaussian* luciferase activity only in androgen-treated Hep3B cells transfected with vectors expressing NH2-terminal and COOH-terminal fragments of *Gaussian* luciferase enzyme linked to AR and JunD, respectively, provides clear and direct evidence of JunD interaction with androgen-activated AR *in situ* (Fig. 3D). Although immunoprecipitation and co-localization of AR with another AP-1 family member, c-Jun, have been reported (31), to the best of our knowledge, this is the first direct demonstration of androgen-activated AR and JunD complex formation.

Because overexpression of JunD is necessary for the induction of ROS following androgen exposure (19, 20), presumably the AR-JunD complex regulates expression of genes involved in ROS production in LNCaP cells. The complex may bind via JunD to sequences containing binding sites for members of the AP-1 family of transcription factors (TGA/C/C/TC; ref. 36). These sequences may or may not contain any ARE sequence. Thus, many genes such as SSAT that are not directly regulated by AR might be regulated by an AR-JunD complex.

By scanning the SSAT gene promoter sequence *in silico*, we identified six putative AP-1-binding sites. Using our sJunD clone of the LNCaP clone cell line (20), we showed that in the absence of JunD, androgen-activated AR does not induce SSAT expression (Fig. 4). Thus, we conclude that androgen-activated AR requires JunD for SSAT expression.

A direct binding of JunD to the SSAT promoter sequence was shown by ChIP assay (Fig. 5). By PCR analysis with primers designed to identify the SSAT promoter, we obtained a PCR product that corresponds to a DNA fragment of the SSAT promoter only in the chromatin fragment precipitated by JunD antibody and not in the chromatin fragment precipitated by AR antibody (Fig. 5A). This suggests that under these conditions, where JunD directly binds to the −574 to −651 bp of the SSAT promoter, there may not be a direct binding of AR to the SSAT promoter. Elucidation of this mechanism also explains the delay in SSAT expression (72 h) and ROS generation after androgen treatment as previously reported (21).

Collectively, our data suggest that activated AR forms a complex with JunD that binds to an AP-1 DNA-binding sequence in the SSAT promoter to activate SSAT gene transcription, resulting in overproduction of H2O2 in CaP cells, as shown schematically in Fig. 6. To the best of our knowledge, the data presented above provide for the first time a molecular mechanism of androgen-induced increase in SSAT activity and consequent ROS overproduction in CaP cells. The demonstration of a mechanistic pathway of androgen-induced ROS production opens up a new avenue for development of drugs that specifically target steps in this ROS-generating pathway in CaP cells and thus can be effective in therapy and prevention of CaP without major systemic toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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