Early Growth Response Gene 1 Modulates Androgen Receptor Signaling in Prostate Carcinoma Cells

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The transcription factor early growth response gene 1 (EGR1) has been implicated in diverse roles in the regulation of cell growth, apoptosis, and differentiation. Previous studies suggest that the effects of EGR1 on tumorigenesis are critically dependent on the cellular context. In a majority of prostate cancers, EGR1 is overexpressed and promotes prostate tumor progression. In contrast, in other tumor types such as breast cancers and glioblastomas, EGR1 is expressed at low levels and when overexpressed can inhibit tumor growth. To explore the role of EGR1 in prostate tumorigenesis, we examined the impact of EGR1 expression on the androgen receptor (AR) signaling pathway. We show here that EGR1 binds to the AR in prostate carcinoma cells, and an EGR1-AR complex can be detected by chromatin immunoprecipitation at the enhancer of an endogenous AR target gene. Overexpression of EGR1 enhanced AR-mediated transactivation, whereas EGR1 knockdown by small interfering RNA inhibited AR signaling pathway activity. Furthermore, Western blot and immunocytochemical analyses showed that constitutive overexpression of EGR1 promotes the translocation of AR from the cytoplasm to the nucleus. These results indicate that EGR1 may promote prostate cancer development by modulating the androgen receptor signaling pathway.

Materials and Methods

Plasmid Constructs—pDNA3.1-EGR1 construct was generated by subcloning an EcoRI/XhoI full-length EGR1 fragment that is resistant to the NAF1-A-binding protein (NAB) corepressors (16) into pDNA3.1 vector. Androgen receptor constructs consisting of pCMV-AR, pGEX-AR1–562 (N-terminal domain), pGEX-AR544–634 (DNA-binding domain), and pGEX-AR624–924 (C-terminal domain) vectors (17) were kindly provided by Drs. F. S. French and J. Tan (University of North Carolina School of Medicine). A modified Renilla luciferase plasmid and the androgen-responsive luciferase reporter gene pARE luciferase plasmid were a gift from Dr. Xu Cao (University of Alabama at Birmingham) (18).

Cell Culture and Stable Transfectants—The androgen receptor-positive human prostate cancer cell line LNCaP and the androgen-independent human prostate cancer cell line DU145 were obtained from ATCC and maintained in RPMI 1640 supplemented with 5% FBS and Dulbecco’s modified Eagle’s medium with 5% FBS, respectively. For the generation of LNCaP cell line stably expressing EGR1, LNCaP cells were plated overnight in 60-mm dishes. The cells were transfected with
pcDNA3.1-EGR1 or pcDNA3.1 control vector using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. The cells were selected to use 800 ng/ml G418 (Mediatech) 48 h after transfection. The resistant clones were pooled 10–14 days later, and the cells were maintained in RPMI 1640 containing 600 ng/ml G418.

Western Blot Analysis—Whole cells lysates were prepared using extraction buffer (50 mM Tris-HCl buffer pH 7.4, 1% Triton X-100, 1% Nonidet P-40, 5 mM CaCl₂, 2 mM phenylmethylsulfonyl fluoride, and 3 mM hydrogen peroxide). Nuclear protein was extracted by the method of Dignam et al. (19). Protein concentrations of supernatants were measured using the Bio-Rad D₆₀ protein assay reagent. Extracts containing 10–20 µg of protein were electrophoresed on a 12% SDS-PAGE gel and blotted onto a Immobilon™-Millipore. The blotted membrane was treated with 5% fat-free dry milk at 4°C overnight and incubated for 2 h at room temperature with the antibodies described below. The membrane was then incubated for 1 h at room temperature with a peroxidase-labeled goat anti-mouse or goat anti-rabbit antibody (Bio-Rad). The membrane was rinsed, treated with ECL reagent (PerkinElmer Life Sciences) for 1 min and exposed to x-ray film at room temperature for 1–3 min. The following antibodies from Santa Cruz Biotechnology were used: rabbit anti-human EGR1 antibody (1:500), mouse anti-human AR N-terminal antibody (1:500), and goat anti-actin antibody (1:1000).

Immunoprecipitation—For immunoprecipitation experiments, DU145 cells were grown in medium with 5% charcoal/dextran-stripped FBS (HyClone) for 24 h and then transfected with pCMV-hAR vector with FuGENE 6 transfection reagent. The cells were treated with 10 nM 5α-dihydrotestosterone (DHT; Sigma) 24 h after transfection. For LNCaP cells, the cells were grown in RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. The cells were treated with 10 nM DHT or vehicle for 1 h before harvest. The cells were lysed in 0.5 ml of extraction buffer. The lysates were incubated on ice for 30 min and subsequently cleared by centrifugation at 12,000 rpm for 15 min at 4°C. The protein concentration of the lysates was determined by Bio-Rad DC protein assay reagent. 10 µl of protein A/G Plus-agarose beads (Santa Cruz) were added to 1.5-mI microtubes containing 250 µl of cellular lysate (1 mg protein/ml) and rotated at 4°C for 1 h. The samples were centrifuged for 1 min at 2000 rpm. Primary antibodies (anti-EGR1, anti-cyclin E (Santa Cruz), or rabbit IgG) and 10 µl of protein A/G Plus-agarose beads were added to the supernatant, and the mixture was rotated overnight at 4°C. The beads were pelleted by gentle centrifugation and washed three times with 1 ml of ice-cold extraction buffer. After the final wash, the precipitated protein complexes were resuspended in SDS sample loading buffer and boiled for 5 min. The samples were centrifuged after vortexing, and the supernatants were analyzed by Western blotting.

Production of GST-AR Fusions—BL21 cells (Amersham Biosciences) transformed with pGEXGST-AR1–562, pGEXGST-AR544–634, and pGEXGST-ARG624–919 plasmids (17) were grown in medium containing ampicillin to an A₆₀₀ value of 0.6–0.8. GST fusion protein expression was induced by adding 1.0 mM isopropyl-1-thio-galactopyranoside (Amersham Biosciences). Bacterial pellets were lysed by sonication in 2 ml of ice-cold phosphate-buffered saline (PBS) containing protease inhibitor mixture. The lysates were mixed 2 h at 4°C with glutathione-Sepharose 4B (Amersham Biosciences). After beads with fusion protein were washed three times with PBS, fusion protein was collected by glutathione elution buffer (Amersham Biosciences) and detected by Western blot using an anti-GST antibody (Santa Cruz).

GST Pull-down Assay—Beads with the GST-AR fusion protein were incubated with nuclear lysates from LNCaP cells. GST-AR-EGR1 complexes were washed with nuclear protein extraction buffer three times, eluted with SDS sample loading buffer, and then analyzed by Western blot.

Dual Luciferase Gene Reporter Experiment—DU145 cells (2.5 × 10⁵) were plated overnight in 6-well plates in RPMI 1640 with 10% regular FBS. The transfection was performed as described by the manufacturer with TransIT-TK® transfection reagent (Mirus, Madison, WI) using EGR1 siRNA or control GFP siRNA (Dharmacon, Lafayette, CO). 48 h after transfection, the cells were harvested. EGR1 expression was evaluated by Western blot analysis. Sequences of EGR1 siRNA and control GFP siRNA are 5′-AGAGGCUAUCAACAGAGUCA-3′ and 5′-GCUCAGUCCAGGAGC-CACC-3′, respectively.

Chromatin Immunoprecipitation (ChIP) Assay—LNCaP cells were grown in RPMI 1640 with 5% charcoal/dextran-stripped FBS for 3 days. The cells were then treated with 10 nM DHT for 1 h and cross-linked with 1% formaldehyde at 37°C for 10 min. The cells were then rinsed three times with ice-cold PBS, collected into PBS with protease inhibitor mixture, and centrifuged for 5 min. The pellets were resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitor mixture, incubated for 10 min on ice, and sonicated six times at 10 s each at 55% input using a Branson Ultrasonics Sonicator (Danbury, CT), followed by centrifugation for 10 min. The supernatants were diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, pH 8.1), followed by immunoclearing with salmon sperm DNA/protein A agarose (Upstate Biotechnology, Inc.). Immunoprecipitation was performed for 2 h at 4°C with rabbit anti-human AR polyclonal antibody (Upstate Biotechnology, Inc.). Rabbit anti-human EGR1 polyclonal antibody, or rabbit IgG. After immunoprecipitation, protein A-agarose was added and incubated overnight. Agarose beads were washed sequentially for 10 min each in low salt wash buffer, high salt wash buffer, and LiCl immune complex wash buffer. The beads were then washed two times (Invitrogen), and 1 µg of total RNA was reverse transcribed. PCR was performed by SYBR® green PCR Master Mix (Applied Biosystems) as described using the relative standard curve method (7). The increase in fluorescence of the SYBR green dye was monitored using a GeneAmp 5700 sequence detection system (Applied Biosystems). All of the PCR reactions were performed in triplicate. The values were normalized to the relative amounts of 18 S mRNA. The sequences of primers used for PCR analyses are as follows: PSA, 5′-CACACCTCCTGGACCTCACAC-3′ (sense) and 5′-GGGAATGACGCCGCAAG-3′ (antisense); 18 S, 5′-CGCCGCTAGAGGTGAAATTCT-3′ (sense) and 5′-CGAAC-CTCGCCATTTGCTG-3′ (antisense).

RNA interference—LNCaP cells (3 × 10⁵) were plated overnight in 6-well plates in RPMI 1640 with 10% regular FBS. The transfection was performed as described by the manufacturer with TransIT-TK® transfection reagent (Mirus, Madison, WI) using EGR1 siRNA or control GFP siRNA (Dharmacon, Lafayette, CO). 48 h after transfection, the cells were harvested. EGR1 expression was evaluated by Western blot analysis. Sequences of EGR1 siRNA and control GFP siRNA are 5′-AGAGGCUAUCAACAGAGUCA-3′ and 5′-GCUCAGUCCAGGAGC-CACC-3′, respectively.
with 10 nM DHT (showing location of AREs. B, GST-AR fusion proteins expression. Fusion proteins were detected by Western blot using anti-GST antibody. The arrows refer to the positions of the respective fusion proteins. C, GST pull-down assay. Beads with the above GST-AR fusions were incubated either in the absence (−) or presence (+) of DHT with LNCaP nuclear extract. GST-AR/EGR1 complexes were eluted by SDS loading buffer and analyzed by Western blot. Input, 5% lysate. NTD, N-terminal domain; DBD, DNA-binding domain; CTD, C-terminal domain.

Fig. 2. EGR1 interacts with the N-terminal domain of AR. A, schematic of GST-AR fusion proteins used and summary of their interaction with EGR1 as determined by GST pull-down assay. B, GST-AR fusion proteins expression. Fusion proteins were detected by Western blot using anti-GST antibody. Input, 5% lysate. NTD, N-terminal domain; DBD, DNA-binding domain; CTD, C-terminal domain. C, schematic of the PSA gene enhancer/promoter showing location of AREs. B, ChIP assay using LNCaP cells treated with 10 nM DHT (+) or vehicle (−) for 1 h. ChIP assay was performed as described under “Materials and Methods” using specific antibodies against AR, EGR1, or control rabbit IgG. Input DNA and protein-bound DNA fragments were amplified by PCR using primers spanning AREIII in the PSA gene enhancer. IP, immunoprecipitation.

RESULTS

EGR1 Binds to the AR in Prostate Carcinoma Cells—In previous studies using recombinant proteins produced in bacteria, EGR1 was shown to interact specifically with the androgen receptor in vitro (20). To determine whether EGR1 interacts with AR in human prostate carcinoma cells, we performed coimmunoprecipitation experiments. The human prostate carcinoma cell line DU145, which does not express detectable levels of AR, was transfected with the pcMV-AR plasmid encoding the full-length human androgen receptor cDNA (17). As shown in (Fig. 1A), a 110-kDa band corresponding to the AR was detected in immunoprecipitates using anti-EGR1 antibody but not in those from control rabbit IgG. The interaction between EGR1 and AR was strongest in cells treated with DHT. This is probably a reflection of the fact that EGR1 is a nuclear protein, and the AR translocation to the nucleus is dependent on DHT. To investigate whether EGR1 binds to endogenous AR in prostate cancer cells, we performed coimmunoprecipitation experiments using LNCaP cells, which express both AR and EGR1. Anti-EGR1 antibody, but not control IgG, coimmunoprecipitated AR in LNCaP cells grown in medium containing normal FBS (Fig. 1B). When grown in the absence of androgens, a weaker but readily detectable interaction between EGR1 and AR was observed in LNCaP cells, and this interac-

Fig. 3. EGR1 binds to AREIII element in the regulatory region of the PSA gene. A, schematic of the PSA gene enhancer/promoter showing location of AREs. B, ChIP assay using LNCaP cells treated with 10 nM DHT (+) or vehicle (−) for 1 h. ChIP assay was performed as described under “Materials and Methods” using specific antibodies against AR, EGR1, or control rabbit IgG. Input DNA and protein-bound DNA fragments were amplified by PCR using primers spanning AREIII in the PSA gene enhancer. IP, immunoprecipitation.

Fig. 4. EGR1 enhances AR-mediated transactivation of ARE luciferase reporter. A, DU145 cells were transfected with human AR expression plasmid, the ARE luciferase reporter, and a Renilla luciferase expression vector for normalization of transfection efficiency. Some samples were also transfected with an increasing dose of an EGR1 expression vector as shown. 24 h after transfection, the cells were treated with DHT (+) or vehicle (−), and luciferase assays were performed an additional 24 h later. The normalized relative luciferase activity is shown. The means and standard deviations of triplicate measurements are shown. B, DU145 cells were transfected with ARE luciferase reporter and with increasing amounts of EGR1 expression plasmid or AR expression plasmid as shown. EGR1 expression alone does not transactivate the ARE luciferase reporter. The AR transfection sample serves as a positive control. The means and standard deviations of triplicate measurements are shown.
to the level of 18S expression. The means and standard deviations of triplicate measurements are shown.

Fig. 5. EGR1 expression activates expression of the endogenous PSA gene. A, EGR1 protein expression in LNCaP cells stably transfected with EGR1 expression vector (Egr1) and control vector (Neo). Actin is shown as loading control. B, LNCaP-EGR1 and LNCaPNeo control cells were cultured in medium with 5% charcoal/dextran-stripped FBS for 3 days. The cells were then treated with DHT (+) or vehicle (−) for 4 h before harvesting. PSA mRNA expression was determined by real time RT-PCR. The expression levels were normalized to the level of 18S expression. The means and standard deviations of triplicate measurements are shown.

Fig. 6. EGR1 overexpression promotes nuclear translocation of AR. A, Western blot analysis of AR protein levels in nuclear and whole cell extracts. LNCaP-EGR1 and LNCaP-Neo cells were cultured in medium with 5% charcoal/dextran-stripped FBS for 5 days. 1 h after exposure to 10 nM DHT, cells were harvested for Western blot. B, AR immunocytochemical assay. LNCaP-EGR1 and LNCaP-Neo cells grown on coverslips were cultured and treated with DHT as in “A” above, then subjected to immunofluorescence with anti-AR antibody (green). Coverslips were mounted with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) to stain DNA (blue).

Fig. 7. EGR1 siRNA down-regulates PSA gene expression in LNCaP cells. A, LNCaP cells cultured in medium containing regular FBS were transfected with siEGR1 or siGFP RNA, and 48 h later, the extracts were prepared for EGR1 and AR Western blot. B, total RNA was prepared from LNCaP cells transfected with siRNA as in A. PSA mRNA expression was determined by real time RT-PCR. The expression levels were normalized to the levels of 18S rRNA expression. The means and standard deviations of triplicate measurements are shown.*, p < 0.05.

EGR1 Binds to N-terminal Domain of AR—To determine which portion of AR interacts with EGR1, we used GST pull-down assays. We expressed and purified GST fusion proteins encoding different domains of the androgen receptor corresponding to the N-terminal domain, the DNA-binding domain, and the C-terminal domain (Fig. 2, A and B). GST pull-down experiments indicate that EGR1 interacts exclusively with the N-terminal activation domain of AR and that this interaction is independent of DHT (Fig. 2C).

EGR1 Binds to Androgen Response Elements (AREs) in the Upstream Domain of the PSA Gene—After translocation to the nucleus, AR forms a complex on the androgen response elements (AREs) present in the regulatory regions of its target genes. To investigate whether EGR1 forms a complex with AR on the AREs of a native promoter in vivo, we used ChIP assays. We examined the interaction of AR and EGR1 on the AREIII element of the well known AR target gene, PSA (Fig. 3A). LNCaP cells were grown in medium containing charcoal/dextran-stripped serum for at least 3 days followed by treatment with 10 nM DHT. Specific antibodies against EGR1 or AR were used to immunoprecipitate bound genomic DNA fragments. As expected, AR antibody precipitated AREIII in a DHT-dependent manner (Fig. 3B). Antibody against EGR1 also precipitated the AREIII element, indicating that EGR1 complexed with AR could interact with a native AR-target gene promoter/enhancer in vivo. Importantly, this interaction is dependent on DHT (Fig. 3B), supporting the conclusion that EGR1 does not directly bind to the AREIII but is recruited through complex formation with AR. Additional control ChIP experiments with normal rabbit IgG further demonstrate the specificity of the interactions.

EGR1 Augments AR-mediated Transcription—To assess the functional significance of the EGR1-AR interaction, we assessed the effect of EGR1 expression on AR-mediated activation of an ARE luciferase promoter-reporter construct. In DU145 cells, transfection of EGR1 and AR in the absence of DHT had a modest stimulatory effect on ARE luciferase reporter activity (Fig. 4A). As expected, DHT treatment stimulated AR activity, and this ligand-dependent transcriptional activity of AR was further enhanced significantly by EGR1 in a dose-dependent manner (Fig. 4A). Expression of EGR1 alone in the absence of AR had no effect on ARE luciferase reporter activity (Fig. 4B). These data indicate that EGR1 augments AR-mediated transcription, and this effect is mediated through the interaction between the two proteins.

EGR1 Expression Enhances Androgen-induced PSA mRNA Expression—To extend the above results to an endogenous AR target gene, we monitored the effect of EGR1 overexpression on the expression of prostate-specific antigen (PSA) mRNA in androgen-dependent LNCaP cells. We first established LNCaP cell lines stably overexpressing EGR1 (Fig. 5A). As shown in Fig. 5B, relative to untreated LNCaP-Neo cells, DHT treatment induced PSA gene expression about 9-fold in LNCaP-Neo cells and ~18-fold in LNCaP-EGR1 cells. Interestingly, LNCaP-EGR1 cells consistently showed elevated expression of PSA even in the absence of DHT (Fig. 5B).

Effect of EGR1 on Nuclear Translocation of AR in LNCaP Cells—The observation that LNCaP-EGR1 cells expressed elevated levels of PSA even in the absence of androgens prompted us to examine whether EGR1 expression can promote the translocation of AR from cytoplasm to nucleus. In the absence of androgens, the AR is localized to the cytoplasm in an inactive complex that includes heat shock proteins. Upon binding to its cognate ligand, the AR undergoes a conformational change that results in a more compact and stable form of the AR. The activated AR dissociates from heat shock protein and translocates to the nucleus where it binds to consensus DNA sequences as a homodimer to influence transcription of down-
stream genes (21). We observed changes in intracellular distribution of AR in LNCaP cells stably expressing EGR1 by Western blot and immunocytochemical analyses. Western blot for nucleated cells from LNCaP and LNCaP-EGR1 after exposure to 10 nM DHT (Fig. 6A). In the absence of DHT, there was a modestly higher level of nuclear protein in LNCaP-EGR1 cells compared with LNCaP-Neo cells. Immunocytochemical analysis confirmed these results. Without DHT, AR protein in LNCaP-Neo control cells was mainly present in the cytoplasm and translocates to the nucleus with DHT treatment (Fig. 6B). In LNCaP-EGR1 cells on the other hand, AR expression could be detected in the nucleus in addition to the cytoplasm in the absence of DHT. Nuclear AR reactivity was further increased upon DHT treatment. These results suggest that EGR1 overexpression can promote translocation of AR from cytoplasm to nucleus.

RNA Interference with EGR1-siRNA Inhibits PSA Expression in LNCaP Cells—To determine whether down-regulation of endogenously expressed EGR1 in prostate carcinoma cells can modulate PSA gene expression, we employed siRNA targeting EGR1. LNCaP cells growing in medium containing 10% regular FBS were transfected with siEGR1 RNA duplexes or control siRNA specific for GFP. It should be noted that regular FBS contains testosterone and DHT, which drive androgen receptor-dependent PSA gene expression. Western blot analysis performed on extracts prepared 48 h after transfection indicates that the siEGR1 leads to a significant reduction in EGR1 protein levels without an effect on AR levels (Fig. 7A). PSA levels were measured by RT-PCR. The results show that EGR1 knockdown by siEGR1 resulted in down-regulation of PSA gene expression 3-fold relative to the expression in siGFP transfected cells (Fig. 7B). These results further confirm that EGR1 expression promotes AR transcriptional activity in prostate carcinoma cells.

DISCUSSION

The androgen receptor signaling pathway plays a crucial role in normal prostate physiology and prostate tumorigenesis (21). Human prostate carcinoma cells are generally androgen-sensitive and react to hormonal therapy by temporary remission, which is invariably followed by relapse to an androgen-independent state. The molecular mechanisms of transition from androgen dependence to androgen independence remain poorly understood, and a number of possibilities have been explored to explain this phenomenon. Earlier theories postulated that androgen-independent cells might not express the AR, or the AR gene could be mutated from androgen-independent tumors to a molecule unable to be activated by androgen. However, several studies show that there is a higher average of AR in androgen-dependent tumors, and the mutation in the AR is present in a low percentage of local recurrence of androgen-independent tumor (22–24). Furthermore, the AR signaling pathway remains active in androgen-independent prostate cancers (21). How are androgen-dependent genes activated in a low androgen medium such as that of patients under maximum androgen blockade? AR coactivators may contribute to the AR activity in androgen-independent prostate cancer. Their overexpression in prostate cancer could make AR signaling pathway be in a superactive state so that AR transcription is activated in a very low androgen concentration (25, 26). There is increasing evidence that signaling pathways of some cytokines and growth factors may activate unliganded AR. They modify AR protein through interactions with AR or AR coactivators, activating AR signaling (27–30).

Our results have identified functional interaction between EGR1 and the androgen receptor. Several studies have implicated EGR1 in prostate tumorigenesis (5, 9, 10). However, in other tumor types such as breast cancer and glioblastoma, EGR1 behaves as a growth suppressor (12, 14). Our findings suggest that the effects of EGR1 in promoting prostate tumorigenesis are mediated, at least in part, by functional interaction with the AR signaling pathway in prostate cells. EGR1 physically interacts with AR and is part of a complex that forms on the regulatory region of endogenous AR target genes. Furthermore, this interaction translates into increased gene expression for the target gene we have examined, PSA. Our in vitro GST pull-down experiments mapped EGR1 interaction to the N-terminal domain of the androgen receptor. Quantitation of PSA expression in LNCaP-EGR1 cells showed that EGR1 modestly enhanced AR-mediated gene activation even in the absence of androgens. This effect is likely due to the fact that significant EGR1 overexpression promotes the translocation of AR from the cytoplasm into the nucleus. It is possible that the outcome of EGR1-AR interaction could differ for other AR target genes. Also, our study has not examined the impact of EGR1-AR interaction on the regulation of EGR1 target genes. This is an interesting area for future investigation.

In summary, we have identified interaction between EGR1 and AR in prostate carcinoma cells. The fact that EGR1 is frequently overexpressed in human prostate cancer makes this interaction potentially significant. We propose that the interaction between EGR1 and the AR signaling pathway may play a part of the prostate-specific pro-tumorigenic effect of EGR1. Further investigation of this interaction will increase our knowledge of context-dependent regulation of tumorigenesis and may help us to identify new steps that can be targeted for prostate cancer therapy.

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