Ligand-independent activation of the androgen receptor by IL-6 and the role of the coactivator SRC-1 in prostate cancer cells

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Running Title: SRC-1 coactivation of the AR N-terminal domain by IL-6

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ABSTRACT

The androgen receptor (AR) can be activated in the absence of androgens by interleukin-6 (IL-6) in human prostate cancer cells. The events involved in ligand-independent activation of the AR are unknown but have been suggested to involve phosphorylation of the AR itself or a receptor-associated protein. Steroid receptor coactivator 1 (SRC-1) has been shown to interact with the human AR, modulate ligand-dependent AR transactivation, and is regulated by phosphorylation by mitogen-activated protein kinase (MAPK). To date no one has examined the role of SRC-1 on ligand-independent activation of the AR by IL-6 or other signaling pathways known to activate the full-length receptor. These studies addressed this and have revealed the following: 1) SRC-1 similarly enhanced ligand-independent activation of the AR by IL-6 to the same magnitude as that obtained via ligand-dependent activation; 2) androgen and IL-6 stimulate the MAPK pathway; 3) MAPK is required for both ligand-dependent and ligand-independent activation of the AR; 4) phosphorylation of SRC-1 by MAPK is required for optimal ligand-independent activation of the AR by IL-6; 5) protein-protein interaction between endogenous AR and SRC-1 is dependent upon treatment of LNCaP cells with IL-6 or R1881; 6) protein-protein interaction between the AR N-terminal domain and SRC-1 is independent of MAPK; and 7) ligand-independent activation of the AR does not occur by a mechanism of solely overexpression of either wild-type SRC-1 or a mutant SRC-1 that mimics its phosphorylated form.
INTRODUCTION

The androgen receptor (AR) is a ligand-mediated transcription factor that belongs to the superfamily of steroid receptors (1). These receptors have similar protein structures that are composed of an N-terminal domain (NTD) that contains activation function-1 (AF-1), a DNA binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) that contains AF-2. After the ligand binds to AR, the heat-shock proteins are dissociated from the AR, the ligand-receptor complex translocates into the nucleus, binds specific androgen response elements (AREs) on the chromosome, interacts with coactivators, and modulates the expression of androgen-regulated genes (2). In the absence of androgen, the AR can be activated by growth factors, interleukin-6 (IL-6), and elevation of intracellular cyclic AMP (3-8). One possible mechanism underlying ligand-independent activation of the AR by these alternative pathways may involve phosphorylation of either the AR itself or receptor-associated proteins such as coactivators (7,8).

Coactivators are proteins that generally do not bind DNA, but are recruited to the promoter through protein-protein interactions with transcription factors such as the AR usually in a ligand-dependent manner. Interaction between the receptor and coactivator enhance receptor-dependent transcription. The first identified member of coactivators that regulate steroid receptor action was steroid receptor coactivator 1 (SRC-1) (9). Phosphorylation of SRC-1 by mitogen-activated protein kinase (MAPK) is required for optimal progesterone receptor-dependent transcription and for functional cooperation with CREB-binding protein (CBP) (10). SRC-1 interacts with both AF-1 and AF-2 of the AR and enhances ligand-dependent transactivation to increase transcription of androgen-regulated genes (11,12).
Prostate-specific antigen (PSA) is an androgen-regulated gene that is routinely used by oncologists and urologists to monitor treatment responses, prognosis and progression in patients with prostate cancer (13). Patients with advanced prostate cancer treated with hormone ablation therapy inevitably succumb to their disease as the tumor progresses to an androgen-independent condition, an early sign of which is a rising titer of serum PSA. There is mounting evidence that ligand-independent activation of the AR may play a role in hormone-refractory disease. This is because the AR is expressed in hormone refractory prostate cancer (14) and many of the same genes that are increased by androgens in androgen-dependent prostate cancer xenografts become elevated in androgen-independent prostate cancer xenografts in castrated hosts (15). PSA gene expression can be induced in prostate cancer cells by IL-6 via ligand-independent activation of the AR (8). Currently the underlying molecular mechanism of ligand-independent activation of the AR is not known. A possible mechanism may include increased expression of coactivators of the AR such as SRC-1 which has been reported to be increased in a large number of recurrent prostate cancer tissue (16,17).

IL-6 has gained considerable clinical interest for a number of reasons including: prostate cancer predominately metastasize to bones that express IL-6 (4,18); epithelial cells from normal, hyperplasia and carcinoma prostate tissue also secrete IL-6 in culture media (19); IL-6 receptor (IL-6R) is expressed in normal prostate, high-grade PIN and cancer (20,21); IL-6 is elevated in the sera of patients with metastatic prostate cancer (19,22,23) and hormone refractory disease (24); and an increase in proliferation of prostate cancer cells has been shown in response to IL-6 (8,25-27) with neutralizing antibody inhibiting the proliferation of PC-3 and DU 145 prostate
In these studies the mechanism of ligand-independent activation of the AR by IL-6 in prostate cancer cells was further investigated. Although much has been reported about the mechanism of ligand-dependent activation of steroid hormone receptors and their interaction with SRC-1, nothing has been reported about the mechanism of ligand-independent activation of the AR and the role of SRC-1. Here we report for the first time that SRC-1 enhances ligand-independent activation of the AR NTD by IL-6 via a pathway that is dependent upon MAPK in LNCaP human prostate cancer cells.
MATERIALS AND METHODS

Cell Culture and Materials - Human prostate cancer LNCaP cells were maintained in RPMI 1640 supplemented with 5% (vol/vol) fetal bovine serum (FBS) (Invitrogen Inc., Burlington, Ontario, Canada), penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37°C in an atmosphere of 5% CO₂. All chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. Bovine serum albumin (BSA) and protease inhibitors (Complete™) were obtained from Roche Diagnostics (Germany). IL-6 was obtained from R&D (Minneapolis, MN). The MEK inhibitor, U0126, was purchased from Promega (Madison, WI).

Plasmids - The human AR cDNA was a gift from Dr. A.O. Brinkman (Erasmus University Rotterdam, The Netherlands). ARR₃-thymidine kinase (tk)-luciferase (31), AR₁-₊五百Gal4DBD, Gal4DBD, p₅Gal4UAS-TATA-luciferase, AR₁₋₂₃₅Gal4DBD were described previously (7,8). PSA (6.1kb)-luciferase was provided by Dr. J.-T. Hsieh (The University of Southwestern Medical Center, Dallas, TX). The expression vectors for SRC-1 were supplied by Dr. B.W. O'Malley and Dr. Nancy Weigel (Baylor College of Medicine, Houston, TX) (10).

Transfection and Luciferase Assay - LNCaP cells (3x10⁵/well) were seeded in 6-well plates and incubated with RPMI 1640 containing 5% FBS for 24 h. Transfection was performed by using Lipofectin Reagent® (5 µl/well) (Invitrogen Inc.) according to the previous published methods (7). The total amount of plasmid DNA was prepared to 3 µg/well by addition of control plasmid that encoded the luciferase gene but lacked the promoter insert. After 24 h, the medium was replaced with serum-free RPMI 1640 containing 1 mg/ml of BSA with R1881, or IL-6. Cells
were collected after 24 h or 48 h incubation using the lysis buffer provided in a luciferase kit (Promega). Luciferase activities were measured by using Dual Luciferase Assay System (Promega) with the aid of a multi-plate luminometer (EG&G Berthold, Germany). Luciferase activities were normalized by the protein concentration of the samples (8,10) as measured by the method of Bradford (35). The results presented as the fold-induction give the relative luciferase activity of the treated cells divided by that of the control. All transfection experiments were carried out in triplicate wells and repeated 3 to 7 times using at least 2 sets of plasmids that were prepared separately.

**Immunoblots** - LNCaP cells (2x10^6/dish) were plated in dishes (10 cm diameter) in RPMI 1640 containing 5% FBS. Twenty-four hours later, the medium was removed and replaced with RPMI 1640 (i.e., serum-free media) for 24 h prior to the addition of IL-6, R1881, or inhibitors. After incubation with these compounds, whole-cell lysates were prepared as described previously (33). Equal amounts of protein (40 µg) from each sample were electrophoresed on SDS-PAGE (7% or 12%) followed by transfer to an Immobilon membrane (Millipore, Bedford) for Western blot analysis. Immunoblots were blocked for 1 h in 5% nonfat dry milk (w/v) in TBST containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20. Blots were incubated with anti-phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody (1:500) (Cell Signaling Technology, Inc., Beverly, MA), anti-SRC-1 antibody (1:200) (Santa Cruz Biotechnology, Inc., CA), anti-AR antibody (AR441, 2 µg/ml, Santa Cruz), and anti-β-actin antibody (Ab-cam, 1:5000, Cambridge, UK), washed with TBST three times, and incubated for 1 h with the second antibody (1:2000). The protein bands were detected by the enhanced chemiluminescence kit (Cell Signaling Technology or Amersham).
**Immunoprecipitation of endogenous AR or AR\textsubscript{N}Gal4DBD fusion proteins** - Endogenous AR was immunoprecipitated from 5x10\textsuperscript{6} LNCaP cells that were plated in T175cm flasks. The cells were incubated in serum free RPMI 1640 media for 24 h before treating with vehicle, R1881 (10 nM) and IL-6 (50 ng/ml) for 6 h. Cells were harvested and nuclear lysates were prepared, precleared with anti-mouse IgG and protein A/G agarose, AR441 antibody (Santa Cruz, 4 µg/ml) was added and the mixture was incubated overnight at 4°C. The immunoprecipitates were washed four times with PBS and re-suspended in sample buffer. The immunocomplexes were separated by SDS-PAGE (7%). Western blot analysis was carried out with anti-SRC-1 antibody (Santa Cruz). AR\textsubscript{N}Gal4DBD fusion proteins were immunoprecipitated from LNCaP cells (2x10\textsuperscript{6}/dish) that were plated in dishes (10 cm diameter) in RPMI 1640 containing 5% FBS for 24 h. Cells transfected with expression vectors encoding SRC-1 (1 µg/dish) and either AR\textsubscript{1-233}Gal4DBD, AR\textsubscript{1-558}Gal4DBD, or Gal4DBD (10 µg/dish) with 60 µl of Lipofectin Reagent® for 24 h were treated with IL-6 (50 ng/ml) or vehicle for 6 h. Cells were harvested and whole cell and nuclear lysates were prepared as described previously (33). After the supernatants of the cells were pre-cleared by adding 0.25 µg of rabbit IgG and 20 µl of protein A/G-agarose (Santa Cruz), anti-Gal4DBD antibody (2 µg; RK5C1; Santa Cruz) was added and the mixture was incubated for 1 h at 4°C. The immunoprecipitates were washed four times with ice-cold lysis buffer and re-suspended in sample buffer. Immune complexes were separated by SDS-PAGE (7%). Western blot analyses were carried out with anti-SRC-1 antibody (1:200; 2 h incubation; Santa Cruz).
RESULTS

Overexpression of SRC-1 enhances androgen-dependent and androgen-independent induction of ARE-driven reporter gene constructs – The role of SRC-1 as a coactivator of the unliganded AR that has been activated by alternative pathways (ligand-independent activation) has not been described. Therefore, we sought to determine if SRC-1 plays a role in ligand-independent activation of the AR by IL-6. To do this, human prostate cancer LNCaP cells that express endogenous AR were transiently co-transfected with ARE-driven reporter plasmids and the expression vector for SRC-1 prior to treatment with synthetic androgen (R1881) or IL-6. The first ARE-driven reporter used was the PSA (6.1kb)-reporter gene construct. This luciferase construct contains both the enhancer and promoter regions of the clinically relevant PSA gene and is induced by androgens (8,34). As shown in Figure 1A, R1881 (10 nM) induced the PSA (6.1kb)-luciferase reporter by approximately 70-fold. Transfection of SRC-1 expression plasmid (0.2 µg) increased this activity to approximately 140-fold, or by 2-fold over that without SRC-1 (compare lane 2 with 6). IL-6 (50 ng/ml) induced the PSA (6.1kb)-luciferase by approximately 17-fold in the absence of androgen (Fig. 1B). Co-transfection of SRC-1 expression plasmid (0.2 µg) increased this activity to approximately 38-fold, or again by approximately 2-fold over that without SRC-1 (compare lane 2 with 6). Basal activity was not altered by enhanced levels of SRC-1 (lanes 1, 3, and 5).

The second ARE-driven reporter used was the ARR3-tk-luciferase, which is an artificial reporter construct that contains three tandem repeats of the rat probasin ARE1 and ARE2 regions upstream of the thymidine kinase promoter (31). Similar to previous reports (8), this promoter was highly inducible by R1881 and in the absence of ectopic SRC-1 was induced approximately
3000-fold (Fig. 1C, lane 2). In the presence of ectopic SRC-1 (0.2 µg), R1881 induced this reporter approximately 8000-fold (lane 6). Consistent with previous reports, IL-6 induction of this reporter was poor in comparison to that achieved with R1881 and only a 6-fold induction was achieved in the absence of ectopic SRC-1 (Fig 1D, lane 2), while approximately 11-fold induction was measured in the presence of ectopic SRC-1 (0.2 µg) (lane 6). Thus, ectopic SRC-1 enhances both androgen-dependent and androgen-independent induction of ARE-driven reporters to a similar extent (2-fold) and these effects were not specific to one particular ARE-driven reporter gene construct.

The effect of inhibiting MAPK activity on IL-6 induction of PSA (6.1kb)-luciferase reporter in the presence of ectopic SRC-1 – MAPK has been suggested to play an important role in androgen-independent prostate cancer (35,36). MAPK also directly phosphorylates SRC-1 at threonine-1179 and serine-1185 (10). To provide insight into the role of MAPK in the enhanced induction of ARE-driven reporters by overexpression of SRC-1, we examined the PSA (6.1kb)-luciferase reporter in LNCaP cells exposed to an inhibitor of MAPK (U0126). Inhibition of MAPK reduced the induction of PSA-luciferase by R1881 both in the presence and absence of ectopic SRC-1 (Fig. 2A). Similarly, inhibition of MAPK reduced the induction of PSA-luciferase by IL-6 both in the presence and absence of ectopic SRC-1 (Fig. 2B). Inhibition of MAPK decreased both androgen-dependent and androgen-independent induction of PSA-luciferase activity in both the presence and absence of ectopic SRC-1. AR protein levels were not altered by treatment of cells with either IL-6 or U0126 (Fig. 2C). AR protein levels were slightly increased in cells treated with R1881 which is consistent with previous reports that ligand stabilizes the AR protein (37).
IL-6 and androgen activate the MAPK pathway - Treatment of LNCaP cells with dihydrotestosterone leads to a rapid and reversible activation of MAPK (38). To test whether the MAPK pathway was being activated in our system with IL-6 and R1881 we performed western blot analyses using antibodies that detect the phosphorylated MAPK isoforms, p44 and p42. Western blot analyses showed that phosphorylation of isoforms p44 and p42 was maximum after 15 minutes of exposure of LNCaP cells to R1881 and IL-6 (Fig. 3, lanes 3 and 5). U0126 completely blocked the phosphorylation of MAPK in cells exposed to R1881 and IL-6 (compare lanes 3 with 4, and 5 with 6). Thus phosphorylation of MAPK was increased in LNCaP cells treated with R1881 or IL-6.

Phosphorylation at threonine 1179 and serine 1185 of SRC-1 is required for optimal stimulation of androgen-independent increases in PSA-luciferase activity by IL-6 - Recently it was shown that MAPK phosphorylates SRC-1 at threonine 1179 and serine 1185 which is required for optimal stimulation of both ligand-dependent and ligand-independent activation of ectopic progesterone receptor in COS cells co-transfected with the GRE2-E1bCAT reporter (10). To date this has not been explored with the AR in context of ligand-dependent or ligand-independent activation. Therefore to determine if phosphorylation of SRC-1 by MAPK is required for androgen-independent induction of PSA by IL-6 via ligand-independent activation of the AR, mutated SRC-1 was compared to wild-type SRC-1 in LNCaP cells. Results presented in Figure 4 show that mutation of threonine 1179 and serine 1185 of SRC-1 to alanines (SRC-1 AA) reduced IL-6-induction of PSA-luciferase activity to a level comparable to that achieved in the absence of ectopic expression of wild-type SRC-1 (compare lane 4 and 2). Mutation of
threonine 1179 and serine 1185 of SRC-1 to glutamic acids (SRC-1 EE) restored SRC-1-enhanced induction of PSA-luciferase activity by IL-6 to a level comparable to that achieved with wild-type SRC-1 (compare lane 5 with 3). Alanine mutations mimic a loss of phosphorylation, while glutamic acid residue mutations maintain a negative charge to mimic phosphorylation (10). Thus, optimal ligand-independent activation of the AR and induction of PSA-luciferase activity by IL-6 requires phosphorylation of SRC-1 at threonine 1179 and serine 1185.

IL-6 activation of the human AR NTD is enhanced by SRC-1 and blocked by inhibition of MAPK – IL-6 activates the AR NTD (8) and SRC-1 interacts directly with the AR NTD in a ligand-independent manner (11,12). Therefore, we sought to determine whether SRC-1a would enhance activation of the AR NTD by IL-6. To do this, the yeast Gal4 system was employed using a chimeric construct of the AR NTD (amino acids 1-558) fused to the Gal4DBD (AR\(_{1-558}\)Gal4DBD) (7,8). LNCaP cells were co-transfected with the expression vector encoding the Gal4DBD, AR\(_{1-558}\)Gal4DBD, SRC-1, and a reporter gene containing the Gal4-binding site (p5×Gal4UAS-TATA-luciferase). As shown in Fig. 5A, IL-6 had a slight effect on the activation of Gal4-luciferase activity in cells expressing only Gal4DBD (lanes 1-6), with or without ectopic SRC-1. IL-6 induction of the Gal4 reporter activity in cells expressing the AR\(_{1-558}\)Gal4DBD protein was greatly enhanced by increased expression of SRC-1 (compare lane 8 with 12). These results show that SRC-1 is required for optimal activation of the AR NTD by IL-6.

To test the effects of MAPK on the activation of the AR NTD by IL-6 in the presence of ectopic SRC-1, the MEK inhibitor, U0126, was employed (Fig. 5B). U0126 had negligible effects upon
basal transactivation of AR\textsuperscript{1-558}Gal4DBD (compare lane 9 with 10). In the presence of IL-6, U0126 reduced Gal4 reporter activity both in the presence (compare lane 15 with 16) and absence of cotransfected SRC-1 (compare lane 13 with 14). Levels of AR\textsuperscript{1-558}Gal4DBD fusion protein were consistent in cells treated with R1881, IL-6, and U0126 (Fig. 5C). These results suggest that activation of the AR NTD by IL-6, in the absence and presence of ectopic SRC-1, is dependent upon the MAPK pathway.

**Phosphorylation at threonine 1179 and serine 1185 of SRC-1 is required for optimal activation of the AR NTD by IL-6** - To determine the role of MAPK phosphorylation of SRC-1 on ligand-independent transactivation of the AR NTD by IL-6, we compared wild-type SRC-1 to the mutant forms (SRC-1 AA and SRC-1 EE) using the Gal4 system. Results presented in Figure 6 show that when threonine 1179 and serine 1185 of SRC-1 were mutated to alanines (SRC-1 AA), SRC-1 failed to increase activation of the AR NTD by IL-6 to the level obtained with ectopic wild-type SRC-1 (compare lane 7 with 6). These mutations in SRC-1 reduced activation of the AR NTD to levels obtained without ectopic expression of wild-type SRC-1 in the presence of IL-6 (compare lane 7 with 5). Mutation of threonine 1179 and serine 1185 to glutamic acids of SRC-1 (SRC-1 EE) restored the synergistic activity of the AR NTD by IL-6 to a comparable level to that obtained using wild-type SRC-1 (compare lane 8 with 6). Thus, optimal ligand-independent activation of the AR NTD by IL-6 requires phosphorylation of SRC-1 at threonine 1179 and serine 1185. Note that in the absence of IL-6, mutation of threonine 1179 and serine 1185 to glutamic acids had no effect on the AR NTD (lane 4), nor did wild-type SRC-1 have any effect without IL-6 (lane 2). This suggests that activation of the AR NTD by IL-6 requires more than solely phosphorylation or enhanced levels of SRC-1.
Protein-protein interaction between endogenous AR and SRC-1. To date all studies examining SRC-1 interaction with the AR have failed to examine the endogenous proteins in whole cells. Thus interaction between SRC-1 and AR was investigated using endogenous complexes isolated from LNCaP prostate cancer cells that were exposed to R1881 and IL-6. These studies revealed that SRC-1 was co-immunoprecipitated with the AR only in cells exposed to R1881 or IL-6 (Fig. 7, lanes 7 and 8). No interaction between the AR and SRC-1 was observed in the absence of these compounds (lane 6).

Functional and physical interaction between SRC-1 and the AR NTD. The region of the AR NTD required for ligand-independent activation and recruitment of SRC-1 by IL-6 has not been reported. Therefore we mapped the region of the AR NTD required for potentiation of IL-6 activation by SRC-1. LNCaP cells were co-transfected with the expression vector encoding the Gal4DBD, AR\textsubscript{1-233}Gal4DBD, AR\textsubscript{1-558}Gal4DBD, SRC-1, and a reporter gene containing the Gal4-binding site (p5×Gal4UAS-TATA-luciferase). Results shown in Figure 8A indicate that IL-6 failed to significantly activate the AR\textsubscript{1-233}Gal4DBD protein either in the presence or absence of ectopic SRC-1 (lanes 5 to 8) as compared to AR\textsubscript{1-558}Gal4DBD protein (lanes 10 and 12). This suggests that either amino acids 234-558 of the AR, or alternatively that the entire NTD (amino acids 1-558) of the AR may be required for biological activity by IL-6 regardless of levels of SRC-1 which are in agreement with previous studies mapping transactivation of the AR by IL-6 to amino acids 234-558 in the absence of ectopic SRC-1 (8).

To test whether amino acids 1-233 physically interacted with SRC-1, co-immunoprecipitation
assays were carried out in LNCaP cells exposed to IL-6. As expected IL-6 induced protein-protein interactions between SRC-1 and AR_{1-558}Gal4DBD in LNCaP cells (Fig. 8B, compare lane 5 with 6). However, inconsistent with transactivation studies (Fig. 8A), IL-6 also induced protein-protein interactions in LNCaP cells that expressed AR_{1-233}Gal4DBD (Fig. 8B, compare lane 3 with 4). No interactions were observed in the absence of IL-6 (lane 3 and 5) or between SRC-1 and Gal4DBD (compare lanes 1 and 2). Inhibition of MAPK did not prevent interaction between SRC-1 and the AR NTD (compare lanes 6 and 7). SRC-1 protein levels were comparable in nuclear extracts prepared from LNCaP cells transfected with Gal4DBD, AR_{1-233}Gal4DBD, and AR_{1-558}Gal4DBD (data not shown). These data suggest that SRC-1 interacts with the AR NTD in LNCaP cells exposed to IL-6 and that this interaction is independent of MAPK activity.
DISCUSSION

Steroid hormone receptors are generally considered to be ligand-activated transcription factors. However, the human estrogen receptor and the chick, rat, and rabbit progesterone receptors can be activated in the absence of cognate ligand by dopamine, growth factors, and compounds that modulate protein kinase pathways (39-43). Similarly, the AR can be activated in the absence of its cognate ligand by growth factors, modulation of protein kinase pathways, and IL-6 (3-8). Ligand-independent activation of the AR has been suggested to be a possible mechanism underlying androgen-independent prostate cancer (7,8,44).

We have previously described ligand-independent activation of the endogenous AR in human prostate cancer cells by IL-6 and compounds that enhance PKA activity via targeting the AR NTD to enhance AF-1 activity (7,8) whereas previously suspected growth factors including EGF, KGF, IGF-I, and IGF-II had no effect (8). The events involved in ligand-independent activation of the AR by alternative pathways are unknown but have been suggested to involve phosphorylation of the AR NTD itself or a receptor-associated protein that interacts with this AR domain. SRC-1 is a coactivator that has been shown to interact with the human AR NTD (11,12), modulate AR transactivation in LNCaP cells (45), and is regulated by phosphorylation by MAPK (10). This together with the recent reports that patients with androgen-independent prostate cancer have elevated levels of SRC-1 (16,17) and MAPK activity (46) in tumor cells, and enhanced levels of IL-6 in their blood (24), provides a rationale for examining the effects of elevated expression of SRC-1 on ligand-independent activation of the AR by IL-6 in prostate cancer cells. These studies have addressed this and revealed the following: 1) SRC-1 enhanced both androgen-independent increases in PSA by IL-6, via ligand-independent activation of the
AR, and ligand-dependent activated AR by 2-fold; 2) androgen and IL-6 stimulated the MAPK pathway; 3) MAPK was required for both ligand-dependent and ligand-independent activation of the AR; 4) phosphorylation of SRC-1 by MAPK was required for optimal ligand-independent activation of the AR by IL-6; 5) protein-protein interaction between endogenous AR and SRC-1 is dependent upon treatment of LNCaP cells with IL-6 or R1881; 6) protein-protein interaction between the AR N-terminal domain and SRC-1 is independent of MAPK; and 7) ligand-independent activation of the AR does not occur by a mechanism of solely overexpression of either wild-type SRC-1 or a mutant SRC-1 that mimics its phosphorylated form.

Steroid receptors show a realignment of helix 12 in the LBD which allows binding to LXXLL motifs of coactivators in the presence of ligand (46,47). Activation function-2 (AF-2) has been mapped to helix 12 (48) and is highly conserved between these receptors. AF-1 is generally considered to be ligand-independent and has been mapped to the NTD of steroid receptors. The relative importance of AF-1 and AF-2 in transcriptional activation by the AR varies depending on the gene and possibly ligand (49). SRC-1 has been shown to interact with both the AF-1 and AF-2 domains of the AR (11,12). Hence the contribution of SRC-1 on the two different AF regions was anticipated to vary depending on the presence or absence of hormone. This was not shown to be the case. Rather, here we show that SRC-1 similarly enhanced both ligand-dependent and ligand-independent activation of the AR by 2-fold as measured by ARE-driven reporter gene constructs. In transactivation studies we show that IL-6 activation of the AR NTD, that contains AF-1, was enhanced by increased expression of SRC-1. Androgens had no effect on the activity of this domain as previously reported (7,8). The observed effect of SRC-1 on the full-length endogenous activated AR was similar regardless of ligand which may indicate a number
of possible mechanisms. One such mechanism may involve the interaction of the NTD with the 
LBD to restrict the binding of SRC-1 to the potential binding sites determined on the AR NTD as 
recently reported (50). Alternatively the fact that the AR is unique from other nuclear receptors 
in the way that AF-1 contributes most, if not all, of the activity to the ligand-bound AR rather 
than AF-2 (51-54), may explain why SRC-1 increased both ligand-dependent and ligand-
Independent transactivation of the AR to the same degree. However, it should be noted that 
previous reports that the AF-1 region of the AR is ligand-independent have been based on 
experiments omitting compounds that are known to activate the full-length receptor in the 
absence of its cognate ligand. Those studies observed that the AR NTD could activate a reporter 
gene in the absence of the LBD to a comparable level to that achieved with the full-length 
receptor in the presence of ligand and thus referred to this as ligand-independent activation via 
the AF-1 region. In order to avoid confusion we will refer to this as constitutive activation of the 
AR NTD since under the same cell conditions the full-length receptor is not activated, and refer 
to activation of the AR NTD by IL-6, which activates the full-length AR in the absence of 
androgens, as ligand-independent activation.

MAPK is elevated in androgen-independent prostate cancer tissue (36) and can activate the AR 
in prostate cancer cell lines (35). The MAPK pathway is activated by both IL-6 and androgen in 
prostate cancer cells (38, Figure 2C). Here we show that inhibition of MAPK activity blocked 
both ligand-dependent and ligand-independent activation of the AR as measured by reduction in 
the activity of ARE-driven reporter gene constructs, regardless of levels of SRC-1. Similarly, a 
decrease in IL-6 induced transactivation of the AR NTD was also achieved by inhibition of 
MAPK. Consistent with these data, mutation of the SRC-1 amino acid residues, known to be
phosphorylated by MAPK, to alanine decreased both the induction of PSA-luciferase and transactivation of the AR NTD by IL-6. Mutation of these residues to glutamic acid, which mimics phosphorylation, restored these activities. Together these data strongly suggest an important role for MAPK in the mechanism of ligand-dependent and ligand-independent transactivation of the AR. It should be stressed that transactivation of the AR was not achieved in the absence of IL-6 with the mutant form of SRC-1 that mimics its phosphorylation state (SRC-1 EE), thereby indicating that ligand-independent activation of the AR requires an additional step to phosphorylation of SRC-1, or enhanced levels of SRC-1. This may explain why R1881 which activates MAPK as shown in these studies does not activate the AR NTD as previously reported (7). Nevertheless the common requirement of MAPK for both ligand-dependent and ligand-independent activation of the AR, indicates that inhibition of MAPK activity may constitute a general mechanism for antagonizing AR function in prostate cancer cells.

There is a consensus in the literature concerning the importance of cell-specific and promoter-specific responses for steroid receptor coactivators yet the majority of work examining coactivators and AR in the context of prostate cancer have used yeast, CV-1, Hela, or COS cells and physiologically non-relevant promoters. The results presented here are the first to show interaction of endogenous AR and SRC-1, as well as interaction between the AR NTD and SRC-1 in human prostate cancer cells. These studies reveal a number of discrepancies with the results obtained using other cells types or methods. In yeast, it was shown that the AR NTD (1-556) and AR-360-494 interact with SRC-1e in a ligand-independent manner (11). Here we show that stimulation of LNCaP cells with IL-6 is required for interaction between SRC-1 and the AR NTD (1-558 and 1-233) and no interaction was detected in its absence. In COS cells, a decrease
in ligand-independent activation of the AR was observed in the presence of increased levels of SRC-1a (11). While in CV-1 cells, enhanced SRC-1a increased constitutive AR-AF-1 (1-555) activity (12). Here we show that in LNCaP human prostate cancer cells SRC-1a slightly enhanced constitutive AF-1 activity, while in the presence of IL-6 (which activates the full-length receptor), SRC-1 markedly enhanced transactivation. The fact that SRC-1 enhanced IL-6 induced transactivation of AR\(_{1-558}\) but not AR\(_{1-233}\) was not due to an inability of SRC-1 to interact with the AR\(_{1-233}\) as shown in immunoprecipitation studies. Rather the inability of SRC-1 and IL-6 to activate AR\(_{1-233}\) was probably due to this region of the AR lacking the essential AF-1 regions required for transactivation (49,51). IL-6 induced protein-protein interaction between the AR NTD and SRC-1 was not prevented by an inhibitor of MAPK in spite of the fact that transactivation was blocked by inhibition of MAPK. This suggests that inhibition of phosphorylation of either SRC-1 or AR NTD does not prevent protein-protein interactions but rather transactivation. This implies that protein-protein interaction between SRC-1 and the AR NTD is independent of MAPK activity. Similarly phosphorylation of threonine 1179 and serine 1185 of SRC-1 does not alter the binding of SRC-1 to the progesterone receptor (10).

In conclusion, we have shown that SRC-1 enhanced both ligand-dependent and ligand-independent transactivation of the AR to a similar extent by a mechanism that is dependent upon MAPK and phosphorylation of SRC-1. Phosphorylation by MAPK was not required for IL-6 induced protein-protein interaction between the AR NTD and SRC-1. Therefore the decrease in transactivation of the AR resulting from inhibition of phosphorylation of SRC-1 may be due to a loss in cooperation between SRC-1 and the CREB-binding protein as described for the progesterone receptor (10). However, an additional mechanism to MAPK phosphorylation of
SRC-1 is required for ligand-independent activation of the AR since a mutation of SRC-1 that mimics phosphorylated SRC-1 was not sufficient to mediate transactivation in the absence of IL-6. Understanding this additional underlying mechanism(s) may help to elucidate the events involved in the progression of prostate cancer to androgen-independence and the biological and clinical significance of ligand-independent activation of the AR by IL-6.
ACKNOWLEDGMENTS

We thank N. Mendelev for excellent technical assistance and Dr. R. Snoek for helpful discussions. This work was supported by the United States National Institutes of Health, George M. O’Brien Research Center Grant P50 DK47656 and the USA Army Medical Research and Materiel Command DOD Prostate Research Program Grant (DAM17-98-1-8577). According to the general terms and conditions set by the US Army Medical Research and Materiel Command, the content of this material does not necessarily reflect the position or policy of the US government and no official endorsement should be inferred.
REFERENCES


239-242


LEGENDS TO FIGURES

Fig. 1. Overexpression of SRC-1 enhances androgen-dependent and androgen-independent induction of the ARE-driven reporter gene constructs. LNCaP cells were transiently co-transfected with PSA (6.1kb)- (A, B), or ARR3-tk-luciferase (C, D) (1 µg/well) with various concentrations of SRC-1a (0, 0.1, and 0.2 µg/well) for 24 h prior to treatment with R1881 (10 nM), IL-6 (50 ng/ml), or vehicle. The total amount of plasmid DNA transfected was normalized to 3 µg/well by addition of the empty vector. After 48 h of incubation with R1881 or IL-6, the cells were harvested and relative luciferase activity was determined. The bars represent the mean ± SE of three separate experiments, each in triplicate.

Fig. 2. The MAPK pathway is involved in SRC-1 coactivation of the AR by R1881 and IL-6 in LNCaP cells. U0126 inhibits ligand-dependent (A) and ligand-independent (B) activation of the AR in LNCaP cells. LNCaP cells were transiently co-transfected with PSA (6.1kb)-luciferase (1 µg/well) with or without SRC-1a (0.2 µg/well) for 24 h and then pre-treated with U0126 (10 µM), or vehicle for 2 h before the addition of IL-6 (50 ng/ml) or R1881 (10 nM) and then incubated for an additional 48 h under serum-free conditions. The bars represent the mean ± SE of three separate experiments, each in triplicate. C, levels of AR protein in LNCaP cells treated with R1881, IL-6, or U0126 for 24 h. Forty µg of protein in whole cell lysates was loaded in each lane and separated by SDS-PAGE, blotted, and analyzed for AR protein and normalized to β-actin.

Fig. 3. Activation of MAPK in LNCaP cells by R1881 and IL-6. LNCaP cells were incubated in serum-free conditions for 24 h and then treated with IL-6 (50 ng/ml), R1881 (10 nM), or
vehicle for 15 min, with or without pretreatment with U0126 (10 µM; 30 min), or vehicle. Phosphorylation of MAPK was detected by Western blot analysis using anti-phospho-MAPK (P-MAPK). Membranes were stripped and re-blotted with anti-MAPK antibody which detected total MAPK (T-MAPK).

Fig. 4. **Phosphorylation of SRC-1 at threonine 1179 and serine 1185 regulates optimal induction of PSA by IL-6.** Mutation of threonine 1179 and serine 1185 of SRC-1 to alanine (SRC-1α AA) reduces SRC-1 coactivation of the AR, while mutation of these residues to glutamic acid (SRC-1α EE) restores the coactivation to levels achieved with wild-type SRC-1α (SRC-1α wt). LNCaP cells were transiently co-transfected with PSA (6.1kb)-luciferase (1 µg/well) with or without pCR3.1 (empty vector), SRC-1α wt, SRC-1α AA, or SRC-1α EE (0.2 µg/well) for 24 h before the addition of IL-6 (50 ng/ml) and then incubated for an additional 48 h under serum-free conditions. The bars represent the mean ± SE of three separate experiments, each in triplicate.

Fig. 5. **Effect of SRC-1 on the activity of the human AR NTD.** A, transactivation assays were performed in LNCaP cells co-transfected with the 5xGal4UAS-TATA-luciferase (1 µg/well) and AR1-558Gal4DBD (50 ng/well), or Gal4DBD (50 ng/well) with or without SRC-1α (0, 0.1, 0.2 µg/well) for 24 h prior to incubation with IL-6 (50 ng/ml), or vehicle for an additional 24 h before harvesting and measuring the relative luciferase activities. B, the effects of inhibition of MAPK on transactivation of the AR assays was examined in LNCaP cells co-transfected with the 5xGal4UAS-TATA-luciferase (1 µg/well), AR1-558Gal4DBD (50 ng/well), or Gal4DBD (50 ng/well) with or without SRC-1α (0.2 µg/well) for 24 h prior to incubation with IL-6 (50 ng/ml),
or vehicle for an additional 24 h. LNCaP cells were pre-treated with the MEK inhibitor, U0126 (10 µM), or vehicle for 2 h prior to the addition of IL-6 (50 ng/ml), or vehicle and then incubated for an additional 24 h before harvesting and measuring the relative luciferase activities. The bars represent the mean ± SE of three separate experiments, each in triplicate. RLU; relative luminescent unit(s). C, levels of AR1-558Gal4DBD fusion protein in LNCaP cells treated with R1881, IL-6, or U0126 for 24 h. Forty µg of protein in whole cell lysates was loaded in each lane and separated by SDS-PAGE, blotted, and analyzed for AR protein using AR441 antibody (Santa Cruz, 2ug/ml). The membranes were stripped and normalized to β-actin protein levels using and antibody to β-actin (Ab-cam, Cambridge, UK, 1:5000).

Fig. 6. **Phosphorylation of SRC-1 at threonine 1179 and serine 1185 regulates optimal transactivation of the AR NTD by IL-6.** Mutation of threonine 1179 and serine 1185 of SRC-1 to alanine (SRC-1a AA) reduces SRC-1 coactivation of the AR, while mutation of these residues to glutamic acid (SRC-1a EE) restores the coactivation to levels achieved with wild-type SRC-1a (SRC-1a wt). Transactivation assays were performed in LNCaP cells co-transfected with the 5xGal4UAS-TATA-luciferase (1 µg/well), AR1-558Gal4DBD (50 ng/well) with or without pCR3.1 (empty vector), SRC-1a wt, SRC-1a AA, or SRC-1a EE (0.2 µg/well) for 24 h prior to incubation with IL-6 (50 ng/ml), or vehicle for an additional 24 h before harvesting and measuring the relative luciferase activities. The bars represent the mean ± SE of three separate experiments, each in triplicate. RLU; relative luminescent unit(s).

Fig. 7. **Physical interaction between the endogenous AR and SRC-1 in LNCaP cells exposed to IL-6.** LNCaP cells were serum-starved for 24 h and then were exposed to R1881 (10 nM,
lanes 4 and 7), IL-6 (50 ng/ml, lanes 5 and 8), or vehicle (lanes 1-3, and 6) for 6 h. Nuclear extracts were precleared with mouse IgG (lanes 3-5), immunoprecipitated with anti-AR antibody (lanes 6-8), and were then analysed by Western blotting (WB) using an anti-SRC-1 antibody. Lane 1: nuclear lysate from non-transfected LNCaP cells. Lane 2: whole cell lysate from LNCaP cells transfected with an expression vector for SRC-1a.

Fig. 8. Interaction between the AR NTD and SRC-1 in LNCaP cells exposed to IL-6. A, Transactivation assays were performed in LNCaP cells co-transfected with the 5xGal4UAS-TATA-luciferase (1 µg/well) and AR$_{1-233}$Gal4DBD (50 ng/well), AR$_{1-558}$Gal4DBD (50 ng/well), or Gal4DBD (50 ng/well) with or without SRC-1a (0.2 µg/well) for 24 h prior to incubation with IL-6 (50 ng/ml), or vehicle for an additional 24 h before harvesting and measuring the relative luciferase activities. The bars represent the mean ± SE of three separate experiments, each in triplicate. RLU; relative luminescent unit(s). B, Co-immunoprecipitation of AR with SRC-1 in LNCaP cells co-transfected with an expression vector for SRC-1a (1 µg/dish) (lanes 1-7) and either Gal4DBD (11 µg/dish; lanes 1 and 2), AR$_{1-233}$Gal4DBD (11 µg/dish; lanes 3 and 4), or AR$_{1-558}$Gal4DBD (11 µg/dish; lanes 5, 6 and 7) and then were exposed to IL-6 (50 ng/ml), or vehicle for 6 h with or without pre-treatment of U0126 (10 µM; 30min) (lane 7). Nuclear extracts were immunoprecipitated with anti-Gal4DBD antibody. Anti-Gal4DBD immunoprecipitates were analysed by Western blotting (WB) using an anti-SRC-1 antibody. IP; immunoprecipitation.
Fig. 1.
**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**Fig. 2.**
Fig. 3.

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- P-MAPK
- T-MAPK

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**Fig. 4.**

Relative luciferase activity (RLU x 10^5/min/mg protein)
**Fig. 5.**

A. Graph showing relative luciferase activity (RLU x 10^6/min/mg protein) for Gal4 protein: Gal4DBD 1-558.

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B. Graph showing relative luciferase activity (RLU x 10^6/min/mg protein) for Gal4 protein: Gal4DBD and AR_{1-558}Gal4DBD.

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C. Western blot images showing proteins R1881, IL-6, U0126, and Actin. The arrow points to AR_{1-558}Gal4DBD.
Fig. 6.

Relative luciferase activity (RLU x 10^8/min/mg protein)

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*Fig. 7.*
**Fig. 8.**

A.

- **Relative luciferase activity (RLU x 10^6/min/mg protein)**

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Gal4 protein: Gal4DBD, AR_1-233Gal4DBD, AR_1-558Gal4DBD

B.

- **Gal4 protein:** Gal4DBD, AR_1-233Gal4DBD, AR_1-558Gal4DBD

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IP: Gal4DBD

WB: SRC-1
Ligand-independent activation of the androgen receptor by IL-6 and the role of the coactivator SRC-1 in prostate cancer cells
Takeshi Ueda, Nasrin R. Mawji, Nicholas Bruchovsky and Marianne D. Sadar
J. Biol. Chem. published online August 5, 2002

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