

Interleukin 6 Activates Androgen Receptor-mediated Gene Expression through a Signal Transducer and Activator of Transcription 3-dependent Pathway in LNCaP Prostate Cancer Cells¹

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Abstract

Interleukin 6 (IL-6) is a cytokine that regulates not only immune and inflammatory responses but also the growth of some tumors, including prostate carcinomas. IL-6 signals through Janus kinase, signal transducer and activator of transcription 3 (STAT3), and mitogen-activated protein kinase and is also able to induce androgen receptor (AR)-mediated gene activation in prostate cancer, which is an important process in prostate cancer androgen-independent progression. We now show that IL-6-induced AR-mediated gene activation requires the activation of STAT3 by IL-6 in LNCaP prostate cancer cells. In particular, STAT3 associates with AR in an androgen-independent but IL-6-dependent manner. Inhibition of STAT3 rather than mitogen-activated protein kinase results in inhibition of AR-mediated gene activation in response to IL-6. These findings not only identify STAT3 as an important signaling molecule required for IL-6-signaling to induce AR-mediated gene activation in prostate carcinoma cells but also reveal the importance of activated STAT3 in human tumor development and progression.

Introduction

Prostate cancer initially occurs as an androgen-dependent tumor, which can be successfully treated with androgen ablation therapy. However, the cancer eventually recurs as an androgen independent tumor and is no longer treatable. Thus, progression from androgen dependence to androgen independence is a critical step in prostate cancer development, but the molecular mechanism for this critical progression is poorly understood. Recent attention has been drawn to the hypothesis that androgen-independent activation of AR³ mediates prostate cancer androgen-independent progression in the absence of androgen. Indeed, amplification of AR or mutations in AR that alter the AR function have been found in a growing number of androgen-independent prostate tumors (1, 2). Recruitment of nonsteroid receptor signaling pathways to activate AR in the absence of androgen has also been reported to contribute to prostate cancer androgen-independent progression (3, 4).

IL-6 is a pleiotropic cytokine that not only regulates immune and inflammatory responses but also regulates the growth of many tumor cells, including prostate carcinoma (5–8). IL-6 as well as other growth factors has been shown to regulate prostate cancer growth and to

activate AR-dependent gene expression in prostate cancer cells in the absence of androgen (4, 9). A growing number of clinical observations have revealed the frequent association of high serum IL-6 levels with androgen-independent prostate tumors (10–12). These findings all indicate that IL-6 is involved in prostate cancer androgen-independent progression. IL-6 receptor is expressed in both prostate cancer tissues and prostate carcinomas cell lines, including the androgen-dependent prostate cancer cell line LNCaP (13). Binding of IL-6 to its receptor leads to activation of JAKs as well as two major downstream signaling components, STAT3 and MAPK (also known as ERK), in LNCaP cells (14, 15). Both MAPK and STAT3 have been reported to mediate signaling cross-talk between steroid receptor and other signaling pathways. MAPK is able to phosphorylate estrogen receptor at serine 118 and leads to its estrogen-independent activation by epidermal growth factor (16). STAT3, upon activation by IL-6, can act as a coactivator of glucocorticoid-bound GR and lead to its synergistic activation by combined treatments with IL-6 and glucocorticoid (17). However, the signaling molecule responsible for IL-6 to induce AR-mediated gene activation in prostate cancer is unidentified. Identification of such a signaling molecule is important for understanding the mechanism of IL-6 signaling in prostate cancer androgen-independent progression. Here we show that IL-6-activated STAT3 associates with AR in an IL-6-dependent but androgen-independent manner and that the activation of STAT3 by IL-6 is required for IL-6 to activate AR in LNCaP cells. Therefore, activated STAT3 is an important signaling molecule for IL-6 to activate AR in prostate carcinoma and may play an important role in prostate cancer progression.

Materials and Methods

Cell Culture, Plasmids, and Transfection. LNCaP cells were maintained as described previously (15, 18). The cDNA of DN-STAT3 (Y705F; Ref. 19) was subcloned into pcDNA3 (Invitrogen). ARE₃-tk-luc was constructed by inserting three tandem copies of ARE from the androgen-responsive, prostate-specific antigen promoter (5'-TGCAAGCAgcaAGTGCTAGC-3') upstream of the tk-luc reporter. tk-luc was created previously by inserting the herpes virus tk promoter (-105/+51) into pGL3 (Promega). JAB, CIS2, and Fib-luc have been described previously (20–22). Transfections were performed using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulfate according to the manufacturer's instructions (Boehringer Mannheim). The stable LNCaP clones were obtained by selection in G418 (600 $\mu\text{g ml}^{-1}$). Expression of either JAB or CIS2 (both contain myc epitope tag) protein was confirmed by using anti-myc antibody.

Immunoprecipitation, Western Blotting, and EMSA. LNCaP cells were serum-starved for 24 h, followed by stimulation with 100 ng ml⁻¹ IL-6 (Pepro Tech, Inc.) for 30 min or for the time indicated. Immunoprecipitation and Western blotting were performed as described previously (15). Anti-phospho-STAT3 or anti-phospho-MAPK antibody (New England Biolabs) was used to detect phosphorylation of STAT3 or MAPK (ERK1 and ERK2), respectively. Total STAT3 or total ERK2 detected with anti-STAT3 or anti-MAPK (ERK2) antibody, respectively, was used as a loading control. EMSA for STAT3 was performed as described previously (23). Cell lysates for immunoprecipitation

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³ The abbreviations used are: AR, androgen receptor; IL-6, interleukin 6; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ARE, androgen-responsive element; tk, thymidine kinase; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; DN, dominant negative.

of AR were prepared as described previously (24), using HKMEN buffer [10 mM HEPES (pH 7.2), 142 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 0.2% NP40, protease inhibitors, and phosphatase inhibitors] to lyse cells 16 h after the indicated treatments.

Reporter Gene Assay. Luciferase reporter construct (250 ng) containing either ARE (ARE₃-tk-luc) or IL-6/STAT3-responsive promoter (Fib-luc) was cotransfected with 1 μg of expression vector as indicated or with pcDNA3 empty vector into LNCaP cells in 12-well plates for 24 h, followed by incubation in serum-free, phenol red-free medium with or without 100 ng ml⁻¹ IL-6, 1 nM R1881 (methyltrienolone; DuPont New England Nuclear) or as indicated for 24 h. Luciferase assays were performed according to the manufacturer's instructions (PharMingen).

Results and Discussion

To investigate the molecular mechanism of IL-6-induced AR activation in prostate cancer cells, we decided to dissect the IL-6 signal transduction pathway in LNCaP prostate cancer cells. We first examined whether IL-6-induced AR-mediated gene activation is dependent on the activation of JAK. This was done by investigating the effect of inhibition of IL-6-induced JAK activation on AR-mediated gene activation in response to IL-6 in LNCaP cells. Inhibition of JAK activation is achieved by overexpression of JAB, which inhibits the JAK2 tyrosine kinase activity by binding directly to its JH1 domain (20). CIS2 is structurally related to JAB but is unable to bind to and inhibit JAK tyrosine kinase (21) and is thus used as a negative control for JAB. ARE₃-tk-luc is a luciferase reporter driven by three copies of ARE placed upstream of the tk promoter (ARE₃-tk-luc) used to detect AR-mediated gene activation. We then transiently transfected LNCaP cells with expression vectors encoding JAB, CIS2, or an empty vector together with ARE₃-tk-luc. In the absence of JAB (vector), treatments of LNCaP cells with IL-6 resulted in about 18-fold stimulation of ARE₃-tk-luc (Fig. 1A), which was comparable to synthetic androgen R1881-mediated ARE₃-tk-luc activation (26-fold; Fig. 1A). Cotreatment of LNCaP cells with IL-6 and R1881 resulted in synergistic ARE₃-tk-luc activation (79-fold; Fig. 1A). Overexpression of JAB specifically inhibited ARE₃-tk-luc activation induced by IL-6 or by a combination of IL-6 and R1881, but it did not interfere with R1881-mediated activation of ARE₃-tk-luc (Fig. 1A). However, overexpression of CIS2 did not affect ARE₃-tk-luc activation in response to either IL-6 or R1881 (Fig. 1A). These data showed that AR-mediated gene activation in response to IL-6 is dependent on and may be downstream of activation of JAK tyrosine kinase.

STAT3 is a major downstream component of the IL-6-JAK signaling pathway. As expected, overexpression of JAB, but not CIS2, strongly blocked IL-6-induced activation of Fib-luc, a luciferase reporter construct used to monitor IL-6/STAT3-mediated gene activation (Ref. 22; Fig. 1B). Fib-luc contains the proximal promoter region of the rat γ-fibrinogen gene that has three IL-6/STAT3-responsive elements. The data confirmed that inhibition of JAK activation resulted in inhibition of STAT3-mediated gene activation in response to IL-6 in LNCaP cells. The fact that STAT3-mediated gene activation by IL-6 or inactivation by JAB parallels that of AR also suggested that there might be a functional correlation between STAT3 activity and AR-mediated gene activation in response to IL-6.

MAPK is another major component of the IL-6 signaling pathway. To further investigate the effect of overexpression of JAB or CIS2 on IL-6-induced phosphorylation and activation of endogenous MAPK as well as STAT3, we have established LNCaP cell lines stably transfected with pcDNA3 empty vector, JAB, or CIS2. As expected, overexpression of JAB completely abolished IL-6-induced activation of ARE₃-tk-luc (data not shown) as well as STAT3 (Fig. 1C) and MAPK (Fig. 1D) phosphorylation, which was detected with antibody that specifically recognizes either the tyrosine 705-phosphorylated

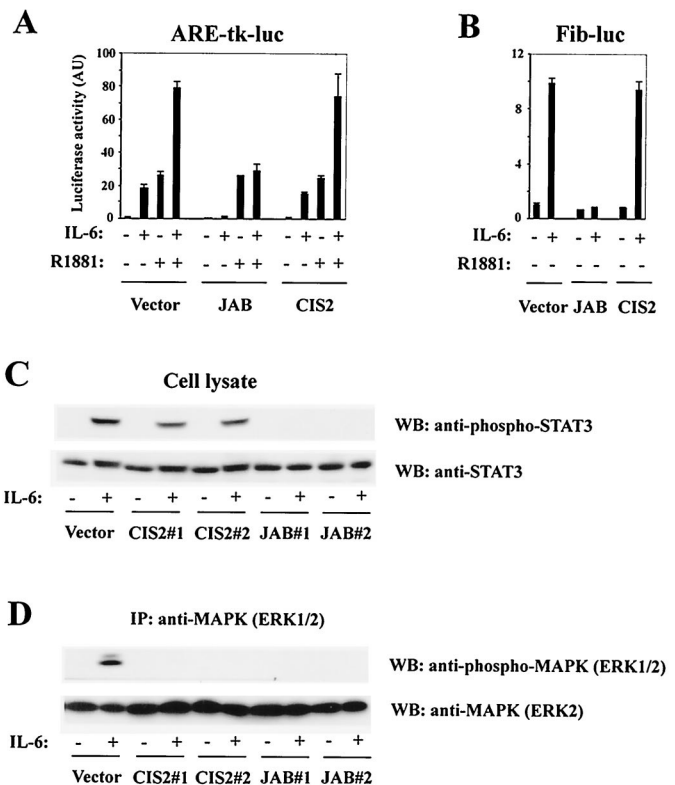


Fig. 1. Inhibition of IL-6 signaling by overexpression of JAB in LNCaP cells blocks activation of AR, STAT3, and MAPK in response to IL-6. For reporter assays (A and B), LNCaP cells were transfected with reporter genes for AR (ARE₃-tk-luc; A) or IL-6/STAT3 (Fib-luc; B) together with either pcDNA3 vector or pcDNA3 vector carrying JAB or CIS2. Luciferase activity (AU, arbitrary units) was measured after the indicated treatments. For detection of MAPK and STAT3 phosphorylation (C and D), LNCaP cells stably transfected with CIS2 (clones 1 and 2), JAB (clones 1 and 2), or pcDNA3 vector were treated as indicated and described in "Materials and Methods". C, anti-phospho-STAT3 (top, active STAT3) and anti-STAT3 (bottom, total STAT3) Western blots of cell lysates. D, anti-phospho-MAPK (ERK1 and ERK2; top, active MAPK) and anti-MAPK (ERK2; bottom, total ERK2) Western blots of anti-MAPK (ERK1 and ERK2) immunoprecipitates.

form of STAT3 or tyrosine/threonine-phosphorylated MAPK (ERK1 and ERK2), respectively. Interestingly, although overexpression of CIS2 had no effect on IL-6-induced STAT3 phosphorylation (Fig. 1C) or ARE₃-tk-luc activation (data not shown), it did inhibit IL-6-induced MAPK phosphorylation through an undefined mechanism (Fig. 1D). This result suggested that MAPK activation might not be required for AR-mediated gene activation in response to IL-6.

Although the mechanism for the inhibitory effect of overexpressed CIS2 on IL-6-induced MAPK activation was undefined, this finding led us to further investigate whether MAPK activation is required for AR-mediated gene activation in response to IL-6. We therefore blocked the activation of MAPK by using the MAPK kinase (MEK1)-specific inhibitor PD 98059. We found that PD 98059, at all of the doses tested (10–50 μM), completely blocked IL-6-induced MAPK phosphorylation (Fig. 2A). This inhibition had no inhibitory effect on either STAT3 tyrosine phosphorylation (Fig. 2B), STAT3 DNA binding as assayed by EMSA (Fig. 2C), or STAT3-mediated gene activation using the Fib-luc reporter (Fig. 2D). Most importantly, PD 98059 did not affect ARE₃-tk-luc activation by IL-6 (Fig. 2E). These results were in agreement with those observed from overexpression of CIS2 (Fig. 1) and confirmed that activation of MAPK is not required for AR-mediated gene activation in response to IL-6.

The above-mentioned finding on MAPK and the strong functional correlation between AR-mediated gene activation and STAT3 activation in response to IL-6 led us to hypothesize that activation of STAT3

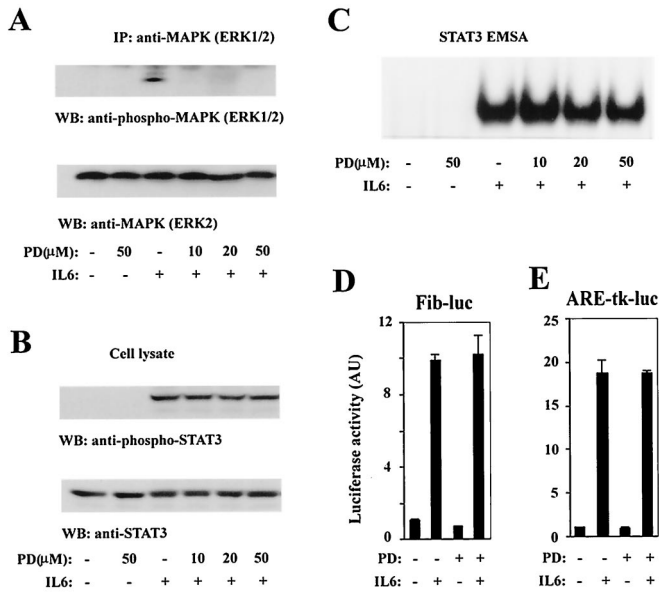


Fig. 2. Inhibition of MAPK has no effect on activation of STAT3 and ARE₃-tk-luc in response to IL-6. A–C, LNCaP cells were pretreated with the indicated dose of PD 98059 (PD) for 15 min, followed by treatment with IL-6 for 30 min. Detection of MAPK phosphorylation (A) and STAT3 phosphorylation (B) was as described in the Fig. 1 legend. C, STAT3 EMSA using a ³²P-labeled oligonucleotide probe corresponding to the *sis*-inducible elements of *c-fos* gene promoter (23). Reporter assays for Fib-luc (D) and ARE₃-tk-luc (E) were performed as described in the Fig. 1 legend. Cells were treated without or with 20 μ M PD 98059 and IL-6 for 24 h after transfection.

might be required for IL-6 to induce AR-mediated gene activation, probably through a functional interaction between AR and STAT3. This hypothesis was first supported by the coimmunoprecipitation of STAT3 with AR in response to IL-6 stimulation in LNCaP cells (Fig. 3A). To detect AR-STAT3 protein-protein interaction at the endogenous protein level, the coimmunoprecipitation assays were performed using lysates prepared from untransfected LNCaP cells. A polyclonal anti-AR antibody or a polyclonal anti-prostate-specific antigen antibody (used as a negative control) was used for immunoprecipitation, and a monoclonal anti-STAT3 antibody was used for subsequent immunoblotting analysis. As shown in Fig. 3A, STAT3 (*top*) was associated with AR immunoprecipitates in an IL-6-dependent manner. However, R1881 failed to either induce the STAT3-AR association by itself or enhance the IL-6-induced STAT3-AR association. Taken together, these data show that IL-6 induces the activation of STAT3 and its subsequent association with AR. However, androgen-AR interaction was not required for the formation of this complex. This observation, combined with the data described above showing that IL-6 alone could activate ARE₃-tk-luc, suggested that androgen-independent activation of AR by IL-6 may use a mechanism different from the one used for androgen-dependent activation of AR.

To further determine the role of IL-6-activated STAT3 in AR-mediated gene activation in response to IL-6, we decided to investigate the effect of a DN form of STAT3 (Y705F-STAT3, in which a phenylalanine was substituted for tyrosine at position 705) on AR-mediated gene activation in response to IL-6. Y705F-STAT3 has been shown to be able to inhibit tyrosine phosphorylation of Tyr⁷⁰⁵ of the wild-type STAT3 and thus inhibit STAT3-mediated gene activation (19). We therefore cotransfected this DN-STAT3 mutant with either ARE₃-tk-luc or Fib-luc reporter into LNCaP cells. As shown in Fig. 3B, overexpression of DN-STAT3 specifically inhibited ARE₃-tk-luc activation in response to IL-6, whereas the response to R1881 was not affected. As a positive control, STAT3-mediated Fib-luc activation in response to IL-6 was also inhibited by DN-STAT3 (Fig. 3C). These

data conformed that activation of STAT3 is required for IL-6 to induce AR-mediated gene activation.

Here we have shown that activation of JAK and subsequently STAT3, rather than MAPK, is required for AR-mediated gene activation in LNCaP prostate carcinoma cells in response to IL-6. These observations represent a novel mode of steroid-independent activation of steroid receptor. Unlike epidermal growth factor-induced estrogen receptor activation (16), MAPK is not required for IL-6-induced AR activation in prostate cancer cells. Unlike the synergistic activation of GR by IL-6 and glucocorticoid, in which IL-6-activated STAT3 only associated with glucocorticoid-bound GR (17), IL-6 alone is sufficient to induce STAT3 activation and its subsequent association with AR in an androgen-independent but STAT3-dependent manner. Therefore, IL-6-activated STAT3 is an important mediator of the IL-6 signaling leading to androgen-independent AR activation. STAT3 may interact with transcriptional coactivators (25) and other signaling molecules, which may be distal to AR but may contribute to AR activation. Thus, in our current model, IL-6 treatment results in phosphorylation and activation of STAT3 and its subsequent association with AR. Recruitment of critical transcriptional coactivators of AR and other signaling molecules by STAT3 to the AR-STAT3 complex could explain the IL-6-induced AR activation in an androgen-independent but IL-6/STAT3-dependent manner.

The clinical observations that elevated IL-6 levels are frequently associated with androgen-independent prostate cancer have predicted an important role for IL-6 signaling in prostate cancer androgen-independent progression. Our finding that IL-6-activated STAT3 is

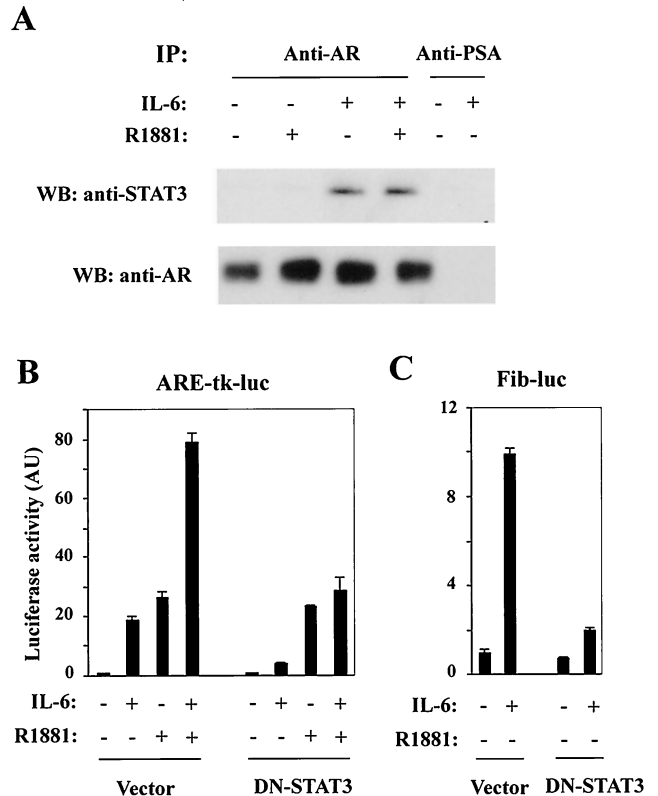


Fig. 3. A, STAT3 coimmunoprecipitates with AR in response to IL-6 in LNCaP cells. Western blotting analysis with either anti-STAT3 (*top*) or anti-AR (*bottom*) was performed on anti-AR immunoprecipitates or anti-prostate-specific antigen immunoprecipitates (negative control). Cells were treated as indicated for 16 h before lysis. B and C, inhibition of STAT3 results in inhibition of ARE₃-tk-luc activation in response to IL-6. LNCaP cells were cotransfected with ARE₃-tk-luc (B) or Fib-luc (C) along with pcDNA3 carrying DN-STAT3 (Y705F-STAT3) or vector alone and then stimulated with or without IL-6 and R1881 as indicated. Reporter assays were performed as described in the Fig. 1 legend.

required for the IL-6 signaling leading to androgen-independent activation of AR in prostate cancer may provide a mechanistic explanation for these clinical observations and is therefore of clinical significance. Recently, activation of STAT3 has been found to mediate IL-6 signaling in the regulation of prostate cancer cell growth (26, 27). Furthermore, our finding also indicates that activated STAT3 may contribute to the development of prostate cancer and possibly other human cancers. Indeed, constitutively activated STAT proteins (very frequently, STAT3) have been found in a growing number of human tumors (28, 29). More recently, Bromberg *et al.* (30) reported that mutationally activated STAT3 can cause cellular transformation and tumor formation and suggested that STAT3 is a proto-oncogene. Therefore, our observation that IL-6-activated STAT3 is required for IL-6 to induce androgen-independent activation of AR in prostate cancer may have broad implications in human cancer development and progression.

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