c-Jun Has Multiple Enhancing Activities in the Novel Cross Talk between the Androgen Receptor and Ets Variant Gene 1 in Prostate Cancer

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Abstract
The multiple transcriptional roles of c-Jun are shown in a novel cross-talk between the androgen receptor (AR) and its new target gene, Ets variant gene 1 (ETV1). In this report, we show that c-Jun can mediate AR induction of ETV1 expression independent of c-Jun transactivation function. Interestingly, c-Jun can transactivate the cloned ETV1 promoter also in the absence of ligand-activated AR, suggesting two mechanisms by which c-Jun can induce ETV1 expression. In addition, both wild-type c-Jun and a transactivation-deficient mutant can enhance the transcriptional activity of ETV1, as measured by both reporter gene assay and endogenous expression of matrix metalloproteinase genes, well-known targets of Ets proteins. Overexpression of the c-Jun mutant protein also led to increased prostate cancer cell invasion. Immunoprecipitation and immunocytochemistry experiments showed copurification and colocalization of c-Jun with AR or ETV1, suggesting that c-Jun acts on AR or ETV1 via a physical association. Collectively, these results, together with a parallel overexpression of ETV1, c-Jun, and AR in prostate tumors, imply that c-Jun plays a pivotal role in the pathway that connects ligand-activated AR to elevated ETV1 expression, leading to enhanced expression of matrix metalloproteinases and prostate cancer cell invasion. (Mol Cancer Res 2007;5(7):725–35)

Introduction
Originally identified as the oncogenic form v-Jun that causes sarcomas in chickens (1), c-Jun represents a protein with multiple transcriptional functions. The best studied and understood function of c-Jun depends on its ability to heterodimerize with c-Fos to form the activator protein-1 (AP-1) transcription factor (reviewed in ref. 2). AP-1 represents a transcription factor complex that is activated by a broad range of external stimuli, including growth factors, chemokines, and the extracellular matrix (reviewed in ref. 3). Through this transactivation process, AP-1 is able to directly induce the expression of a wide variety of genes, many of which mediate cell cycle progression (reviewed in ref. 2). A second transcriptional function of c-Jun is transrepression, a process by which c-Jun is able to interfere with the activities of other transcription factors, including nuclear receptors (reviewed in ref. 4). Coactivation is a third major function for c-Jun that, like transactivation, positively regulates transcription. However, unlike transactivation, coactivation does not depend on dimerization with Fos family members (5) or direct DNA binding (6), and has been best characterized on the androgen receptor (AR), a nuclear receptor that mediates the biological activities of androgens (7).

Among the multiple physiologic functions of the AR, its important role in prostate cancer has been a focus of both basic and clinical research. The development of prostate cancer, just like the normal prostate gland, is dependent on androgen signaling through the AR. In addition, prostate cancer progression is dependent on androgens and AR (reviewed in ref. 8). Indeed, it was recently shown that progression to hormone-refractory prostate cancer, a lethal form of the disease, is induced by the overexpression only of AR (9), a genetic change that is often seen in late-stage prostate cancer. Interestingly, AR transcriptional functions remain important as the cancer cells transition from a hormone-dependent to a hormone-independent stage, and the AR adapts to be activated by mechanisms that do not depend on androgens (8).

AR has several supportive functions in prostate cancer, including inhibiting apoptosis and inducing cell proliferation and invasiveness (8). Several AR-regulated genes have been identified that mediate the antiapoptotic and proproliferative effects of this nuclear receptor (10). We have recently identified Ets variant gene 1 (ETV1) as a novel androgen-regulated gene that mediates prostate cancer cell invasion (1). ETV1 is a member of the Ets family of transcription factors (reviewed in ref. 11). Ets proteins have been reported to be involved in intestinal tumors (12), gastric cancer (13), and breast cancer metastasis (14, 15). Most interestingly, ETV1 and its Ets relative ERG were recently identified to be targets for translocation with TMPRSS2, an androgen-regulated gene, in a subset of prostate cancers (16). Our previous data show that ETV1 androgen regulation or overexpression in prostate tumors does not depend on the TMPRSS2 translocation (1), suggesting that the intact ETV1 plays an important role in prostate cancer.

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In this report, we show that AR induction of ETV1 gene expression in prostate cancer cells is mediated by c-Jun coactivation. Interestingly, the c-Jun enhancement activity was also observed on ETV1 transcriptional activity, as measured by both reporter gene assay and endogenous gene expression, leading to elevated prostate cancer cell invasion. Using immunoprecipitation and immunocytochemistry studies, we can detect a physical interaction and a nuclear colocalization between endogenous c-Jun and AR or ETV1 in prostate cancer cells. Lastly, ETV1 and AR overexpression in prostate tumors is paralleled by overexpression of c-Jun, suggesting coordinated expression patterns among these three proteins in prostate cancer.

Results
c-Jun Coactivation Enhances Androgen-Induced Expression of ETV1

Our previous results show that c-Jun can act as a coactivator for AR transcriptional activity (17, 18) and androgen-dependent gene expression (19). We have recently identified ETV1 as a novel androgen-regulated gene.1 To determine if changes in endogenous c-Jun expression levels could affect androgen induction of ETV1 expression, LNCaP stable cell lines were used that express either antisense c-Jun (AJ81 cells) or c-Jun(Ala63/73) (M37 cells; ref. 19). We have previously shown that AJ81 cells have greatly reduced endogenous c-Jun expression and androgen-dependent proliferation, whereas M37 cells express c-Jun(Ala63/73), a mutant of c-Jun that is fully active in AR coactivation but deficient in AP-1 transactivation, and display significantly elevated proliferation (19). As shown in Fig. 1A, dihydrotestosterone (DHT)-induced expression of ETV1 mRNA is markedly enhanced in M37 cells as compared with C14 cells, which are stably transfected with an empty vector (19). In contrast, the DHT effect on ETV1 expression is almost completely eliminated in AJ81 cells, which have drastically reduced levels of endogenous c-Jun (19). These data were confirmed by quantitative PCR, which showed that
DHT-induced levels of ETV1 are significantly elevated in M37 cells and inhibited in AJ81 cells, as compared with C14 cells (Fig. 1B). Western blotting was used to measure the expression of ETV1 protein, showing higher expression in M37 cells and lower expression in AJ81 cells than in C14 cells (Fig. 1C). To determine if the increased ETV1 expression in M37 cells is due to the elevated levels of Jun protein in these cells, c-Jun small interfering RNA (siRNA) was transfected into M37 cells, resulting in reduced endogenous c-Jun expression (both wild-type and mutant; Fig. 1D). Importantly, the c-Jun siRNA also resulted in a marked reduction in DHT-induced ETV1 mRNA (Fig. 1E) and protein (Fig. 1D) expression. Collectively, these data suggest that the c-Jun coactivation function on AR is an important regulator of ETV1 expression in prostate cancer cells.

To determine if the c-Jun(Ala63/73) mutant protein overexpressed in M37 cells is able to directly transactivate ETV1 overexpressed in M37 cells, showing higher expression in M37 cells and lower expression in AJ81 cells than in C14 cells (Fig. 1B). Western blotting was used to measure the expression of ETV1 protein, showing higher expression in M37 cells and lower expression in AJ81 cells than in C14 cells (Fig. 1C). To determine if the increased ETV1 expression in M37 cells is due to the elevated levels of Jun protein in these cells, c-Jun small interfering RNA (siRNA) was transfected into M37 cells, resulting in reduced endogenous c-Jun expression (both wild-type and mutant; Fig. 1D). Importantly, the c-Jun siRNA also resulted in a marked reduction in DHT-induced ETV1 mRNA (Fig. 1E) and protein (Fig. 1D) expression. Collectively, these data suggest that the c-Jun coactivation function on AR is an important regulator of ETV1 expression in prostate cancer cells.

To determine if the c-Jun(Ala63/73) mutant protein overexpressed in M37 cells is able to directly transactivate ETV1, we used an ETV1-Luc reporter plasmid in a transfection experiment in LNCaP cells. This reporter plasmid contains a 1-kb ETV1 promoter fragment that is activated by AR and is able to recruit AR in a ligand-dependent manner. As shown in Fig. 1F, transfected wild-type c-Jun was able to strongly activate the cloned ETV1 promoter, whereas c-Jun(Ala63/73) was almost completely inactive. Western blotting confirmed similar expression of the two c-Jun proteins. This finding suggests that the increased ETV1 expression in M37 cells is likely due to elevated AR activity in response to c-Jun coactivation. It is interesting to note, however, that c-Jun(Ala63/73) can weakly enhance ETV1 mRNA expression in absence of DHT (Fig. 1B), whereas it has no effect on the ETV1 promoter under the same conditions (Fig. 1F), suggesting that the endogenous ETV1 promoter may harbor an element(s) through which c-Jun(Ala63/73) can act independent of ligand-activated AR, and this is absent from the 1-kb ETV1 genomic fragment found in the ETV1-Luc reporter plasmid.

ETV1 transcriptional activity was also measured using the ETV1-regulated luciferase (Luc) reporter plasmid Fes3×WT-Luc, which contains three copies of an Ets-responsive element from the Fes promoter (20). This promoter was strongly induced by transfected ETV1 in LNCaP cells. When it was tested for DHT regulation in the LNCaP cell lines, the transfected Fes promoter exhibited a small but reproducible DHT-induced increase in activity (Fig. 1G). Importantly, this promoter yielded higher DHT induction in M37 cells than in C14 cells and no DHT effect in AJ81 cells (Fig. 1G). Interestingly, M37 cells exhibited significantly higher Fes promoter activity than LNCaP cells in the absence of DHT. These data suggest that DHT-activated AR can induce the activity of an ETV1-regulated reporter plasmid and that the c-Jun coactivation function may be important in this effect.

We have previously published that the androgen-induced expression of prostate-specific antigen (PSA), hKLK2, and TMPRSS2 is elevated in M37 cells as compared with C14 cells (19), suggesting that c-Jun can mediate AR transactivation of these androgen-regulated genes. The gene microarray approach was used not only to identify ETV1 as a novel androgen-regulated gene (21) but also to measure the potential role of c-Jun coactivation on other androgen-regulated genes. This analysis showed significantly increased androgen-induced expression in M37 cells, as compared with C14 cells, for some genes including the three genes mentioned above (data not shown) and VEGF (Table 1). Interestingly, other genes, including soluble guanylyl cyclase c1 (sGCc1; ref. 21), cyclin B, promyelocytic leukemia zinc finger (PLZF), and Nkx3.1, were not altered in their expression (Table 1). These data show that c-Jun coactivation of AR-regulated gene expression is gene specific.

### c-Jun and AR Coassociate and Colocalize in LNCaP Cells

Our data above show that c-Jun stimulates AR transactivation of ETV1. To determine if there is a physical association between c-Jun and AR, immunoprecipitation experiments were done with endogenous proteins found in LNCaP cells. When an anti-c-Jun antibody was used to immunoprecipitate endogenous c-Jun in LNCaP cells, c-Jun and AR proteins were copurified (Fig. 2A). Neither c-Jun nor AR was purified with a nonspecific immunoglobulin G (Fig. 2A). These findings show an endogenous interaction between c-Jun and AR in LNCaP cells and confirm previous data (17).

Our success with the immunoprecipitation experiments above in LNCaP cells prompted us to investigate subnuclear colocalization of endogenous AR and c-Jun. This was done by immunofluorescence with anti-AR monoclonal and anti-c-Jun

#### Table 1. Expression of ETV1 and Other Androgen-Regulated Genes in LNCaP Cells Using Oligonucleotide Gene Microarray Analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>DHT-Induced Expression</th>
</tr>
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<tbody>
<tr>
<td><strong>C14</strong></td>
<td><strong>M37</strong></td>
</tr>
<tr>
<td>ETV1 (U17163)</td>
<td>708 (P = 0.0002)</td>
</tr>
<tr>
<td>VEGF (M63978)</td>
<td>899 (P = 0.0001)</td>
</tr>
<tr>
<td>sGCc1 (Y15273)</td>
<td>1154 (P = 0.000001)</td>
</tr>
<tr>
<td>Cyclin B (M25753)</td>
<td>1245 (P = 0.00074)</td>
</tr>
<tr>
<td>PLZF (AF060568)</td>
<td>500 (P = 0.000001)</td>
</tr>
<tr>
<td>Nkx3.1 (U80669)</td>
<td>374 (P = 0.0011)</td>
</tr>
</tbody>
</table>

NOTE: LNCaP (C14) and M37 cells were grown for 2 d in the presence (+) or absence (−) of 100 nmol/L DHT. I (increase), D (decrease), and NC (no change) represent the effect of DHT on gene expression in the same cell line (C14 or M37) or comparison of C14 and M37 cells in the presence of DHT.

polyclonal antibodies. As shown in Fig. 2B, androgen treatment stabilized endogenous AR and caused nuclear localization in LNCaP cells, as previously reported (22). Just as with ligand-activated AR, endogenous c-Jun is also nuclear (Fig. 2C).

When the two immunofluorescence images were merged, a fraction of AR was found to colocalize with c-Jun, as shown by yellow staining. These results show an in vivo nuclear colocalization between AR and c-Jun. To determine if the changes in c-Jun expression alter the colocalization with AR, M37 cells were also studied. As shown in Fig. 2C, the expression of c-Jun(Ala63/73) led to increased intensity of the c-Jun signal in M37 cells as compared with C14 cells, whereas AR levels were unaltered. Importantly, when the c-Jun and AR images were merged, there is significantly more nuclear colocalization of these two proteins in M37 cells than in C14 cells (Fig. 2C), strongly suggesting that the overexpressed c-Jun(Ala63/73) can colocalize with ligand-activated AR. These data collectively show that endogenous c-Jun colocalizes with endogenous AR in LNCaP nuclei and this colocalization is directly proportional to the levels of c-Jun protein.

To more closely examine the colocalization of AR and c-Jun in M37 cells, the confocal image of one cell was amplified. As Fig. 2D shows, AR and c-Jun are colocalized on multiple nuclear sites, suggesting that these two proteins act together on multiple genomic sites. This is consistent with our microarray results (Table 1) and reverse transcription-PCR (RT-PCR; ref. 19) results, showing that c-Jun can coactivate multiple AR-regulated genes. Our data show that VEGF and ETV1 exhibit elevated expression in M37 cells, whereas other genes including Nkx3.1 are not changed in their expression (Table 1). Our confocal images also show that AR and c-Jun are found on other sites without colocalization (Fig. 2D), suggesting that these two transcription factors can also act independent of one another, as would be expected on the Nkx3.1 genomic site as well as others.

To determine if c-Jun interaction with AR can lead to recruitment of c-Jun to the ETV1 promoter, chromatin immunoprecipitation was used. In our earlier work, we have shown by chromatin immunoprecipitation that liganded AR was recruited to an ETV1 promoter region harboring a putative androgen-responsive element. The chromatin immunoprecipitation assay was repeated to determine if c-Jun can be co-recruited to this same ETV1 promoter region. The recruitment of c-Jun to the ETV1 promoter is weak in the absence of DHT and, importantly, markedly stronger in the presence of DHT, especially at 24 h (Fig. 2E). Because PSA gene expression is also enhanced by c-Jun coactivation (19), the same chromatin immunoprecipitation experiment showed androgen-induced

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**FIGURE 2.** Endogenous c-Jun and AR coassociate and colocalize in LNCaP cells. **A.** Whole-cell extracts were prepared from LNCaP cells grown in 10% serum and subjected to immunoprecipitation (IP) with an anti-c-Jun antibody. Western blotting was used to detect AR (top) or c-Jun (bottom). The negative control immunoprecipitation was done with an immunoglobulin G (IgG) antibody. “Input” represents 20% of extract used in the immunoprecipitation experiments. **B** to **D.** Immunocytochemistry was used to measure subcellular localization of AR in LNCaP cells treated with 100 nmol/L DHT (+) or ethanol (−; B) or AR and c-Jun in LNCaP stable cell lines treated with 100 nmol/L DHT (+) or ethanol (−; C) or AR and c-Jun in LNCaP stable cell lines treated with 100 nmol/L DHT (+) or ethanol (−; D). Magnification, ×400 (B and C); ×1,000 (D). **E.** Chromatin immunoprecipitation assay was done with LNCaP cells treated with or without DHT for the indicated times to measure c-Jun recruitment to the ETV1 1-kb promoter, PSA promoter, or sGCα1 promoter.

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binding of c-Jun to the PSA promoter that was similar to the ETV1 promoter (Fig. 2E). As a negative control, we used the promoter for sGCα1, whose expression is not affected by c-Jun (see Table 1). Whereas the sGCα1 promoter can recruit liganded AR (21), no c-Jun recruitment was detected (Fig. 2E). These results suggest that c-Jun is recruited to only those AR-regulated promoters that respond to c-Jun coactivation and that this c-Jun recruitment depends on ligand-activated AR.

**c-Jun Regulates the Expression of ETV1-Induced Matrix Metalloproteinase Genes**

Because previous evidence suggests that c-Jun can stimulate the activity of PEA3 proteins (12, 23), we hypothesized that c-Jun can enhance ETV1 transactivation. This hypothesis was first tested using the ETV1 reporter plasmid Fes3×WT-Luc (A). As shown in Fig. 3A, transfected ETV1 or c-Jun similarly induced (~10-fold) Fes promoter activity, whereas the c-Jun(Ala63/73) mutant was weaker. However, this c-Jun mutant was able to strongly enhance ETV1 activation of this promoter, with an activity similar to wild-type c-Jun (Fig. 3A). Similar but weaker effects were seen on matrix metalloproteinase 1 (MMP1)-Luc (Fig. 3B), a reporter plasmid driven by the MMP-1 gene promoter (24). Hence, c-Jun enhances ETV1 transactivation independent of promoter specificity.

The MMP-1 promoter harbors a consensus AP-1 site (25), whereas the Fes promoter does not. J-L. Baert, personal communication.

FIGURE 3. c-Jun stimulates the activities of ETV1-regulated promoters. LNCaP cells were transfected with expression plasmids for ETV1, c-Jun, or c-Jun(Ala63/73) in different combinations and Fes3×WT-Luc (A), MMP1-Luc (B), or MMP1(mAP1)-Luc (C). D. The same transfection as in (B) except that c-Jun(M14) mutant was used in place of c-Jun(Ala63/73) and the AP-1-responsive reporter plasmid TREP3-tk-Luc (19) was also used. E. LNCaP cells were transfected with c-Jun or c-Jun(Ala63/73) and control or ETV1 siRNA together with MMP1-Luc. In all cases above, “Vector” is transfection with empty vector as a control and the luciferase activity is represented relative to the activity of Vector, and this activity was set to 1. F. Different LNCaP stable cell lines were transfected with MMP-Luc or Fes3×WT-Luc reporter plasmids. The luciferase activity is represented relative to the activity in C14 cells, and this activity was set to 1. *, P < 0.05, statistical significance in the negative effect of ETV1 siRNA (E) or of luciferase activity in M37 cells (F).
overlapping nature of the ETV1 and AP-1 binding sites (25). Therefore, we used an alternative approach, in which the c-Jun mutant M14, deficient in dimerization and thus DNA binding (26), was used. As shown in Fig. 3D, M14 is able to cooperatively activate the wild-type MMP-1 promoter as well as wild-type c-Jun protein, whereas it has no activity on an AP-1 responsive promoter (Fig. 3D), showing that the cooperation of c-Jun with ETV1 does not depend on the DNA-binding function of c-Jun.

To show that the c-Jun enhancement of the activity of the ETV1-regulated promoter relies on the endogenous ETV1 in LNCaP cells, we cotransfected c-Jun or c-Jun(Ala63/73) mutant together with ETV1 siRNA to block endogenous ETV1 expression (Fig. 3E). ETV1 siRNA significantly attenuated the c-Jun positive effect on the MMP-1 promoter, suggesting that c-Jun depends on endogenous ETV1 for activation of the MMP-1 promoter. To further confirm these results, we used our two LNCaP stable cell lines that express either c-Jun(Ala63/73) mutant (M37 cell line) or antisense c-Jun (AJ81 cell line). As seen in Fig. 3F, M37 cells had significantly higher activity from both the MMP-1 and Fes promoters than did C14 cells, whereas AJ81 cells had markedly lower activity. Collectively, these results show that c-Jun can cooperate with ETV1 to transactivate ETV1-regulated promoters independent of c-Jun transactivation function.

Next, we studied the importance of c-Jun on endogenous MMP gene expression. As shown in Fig. 4A, transfecting LNCaP cells with either ETV1 or c-Jun(Ala63/73) significantly induced expression of MMP-1, consistent with the promoter assays in Fig. 3B, and MMP-9, whereas MMP-7 and MMP-13 expression was only weakly increased in M37 cells as compared with C14 cells (Fig. 4B). To examine if the elevated expression of MMP-1 and MMP-9 in M37 cells was due to c-Jun cooperation with endogenous ETV1, we used siRNA to specifically reduce ETV1 or c-Jun protein levels. As expected, transfection with ETV1 siRNA significantly decreased MMP-1 and MMP-9 expression (Fig. 4C), indicating an essential transactivation role for ETV1 in M37 cells. The siRNA effects on ETV1 expression in M37 cells were confirmed by RT-PCR (Fig. 4C) or Western blotting (Fig. 4E).

The reduction of endogenous c-Jun by siRNA also resulted in decreased levels of MMP-1 and MMP-9 (Fig. 4D), supporting our hypothesis that the expression of c-Jun(Ala63/73) mutant in M37 cells is responsible for the increased ETV1 transactivation in these cells. AJ81 cells, which have reduced levels of c-Jun (9), expressed markedly lower MMP-7 and MMP-13 mRNA levels (Fig. 4F), consistent with a cooperative function of endogenous c-Jun on ETV1 in LNCaP cells.

c-Jun Associates and Colocalizes with ETV1

Our data above show that c-Jun stimulates both AR transactivation of ETV1 and subsequent ETV1 transactivation of MMP genes, indicating that c-Jun can cooperate with both transcriptional activators. To determine if there is a physical association between c-Jun and ETV1, immunoprecipitation experiments were done with endogenous proteins found in LNCaP cells. When ETV1 was immunoprecipitated with an anti-ETV1 antibody that has been successfully used before (15), copurified c-Jun was detected by Western blotting (Fig. 5A). The negative control immunoprecipitation did not copurify c-Jun (Fig. 5A). Immunoprecipitated ETV1 could not be monitored in this immunoprecipitation experiment because the antibody heavy chain comigrates on the SDS-PAGE gel with ETV1.2 This demonstration of a physical association between ETV1 and c-Jun supports the c-Jun cooperative function.

Colocalization between c-Jun and ETV1 was measured by immunocytochemistry in LNCaP cells lines, just as it was done for c-Jun and AR (see Fig. 2). This experiment showed a colocalization of c-Jun and ETV1 in the nuclei of LNCaP cells (Fig. 5B). The magnitude of colocalization depends on c-Jun levels because c-Jun/ETV1 colocalization is greatly enhanced.

FIGURE 4. c-Jun stimulates ETV1 transactivation of MMP genes. Semiquantitative RT-PCR was used to measure the expression of MMP genes in LNCaP cells transfected with either c-Jun(Ala63/73) or ETV1 (A), C14 or M37 cells (B), M37 cells transfected with ETV1 siRNA (C) or c-Jun siRNA (D), or C14 and AJ81 cells (E). Western blotting was used to show reduced expression of ETV1 in M37 cells transfected with ETV1 siRNA.
in M37 and inhibited in AJ81 cells, when compared with C14 control cells (Fig. 5B). Together, these data suggest that c-Jun can mediate ETV1 transactivation by associating with ETV1 in the nuclei of prostate cancer cells.

**c-Jun Mediates Invasion of Prostate Cancer Cells**

Our data above (see Fig. 4) show that exogenous expression of c-Jun or c-Jun(Ala63/73) led to elevated MMP gene expression. These data, together with our recent finding that ETV1 mediates LNCaP cell invasion, suggest that c-Jun may mediate cell invasion. This hypothesis was tested by comparing the invasive capacity of M37 cells with that of C14 cells. As shown in Fig. 6A, DHT has a small but reproducible positive effect on C14 cell invasion, as we have previously determined. Interestingly, this invasive ability is significantly elevated in M37 cells, both in the absence and presence of DHT (Fig. 6A), showing that expression of c-Jun(Ala63/73) enhances the invasive capacity of LNCaP cells.

**c-Jun Overexpression in Prostate Cancer Tumors Parallels ETV1 Expression**

Our previous data show that ETV1 mRNA and protein are overexpressed in prostate cancer tissues. Further, we determined that this overexpression occurs independent of the translocation with the TMPRSS2 gene that was recently reported. If c-Jun is important in ETV1 activity, as our data suggest in this study, then c-Jun expression in prostate cancer tissues may parallel the expression of ETV1. This was tested by semiquantitative RT-PCR of prostate tissues purchased from the Cooperative Human Tissue Network. Cooperative Human Tissue Network provides tissues that are normal, benign prostatic hyperplasia, or malignant prostate cancer. To confirm that these tissues represent different stages of prostate cancer, the expression of several other genes was measured. This included PSA, which is highly expressed in both malignant prostate cancer and several benign prostatic hyperplasia tissues; EZH2, which is only expressed in the two malignant prostate cancer samples; and E-cadherin, whose expression is significantly reduced in the benign prostatic hyperplasia and malignant prostate cancer tissues as compared with normal. ETV1 mRNA is highly expressed in the two malignant prostate cancer samples and one benign prostatic hyperplasia (Fig. 6B); no ETV1 was detected in the one normal tissue (Fig. 6B). Interestingly, c-Jun expression closely parallels the expression of ETV1, with the highest levels in the two malignant prostate cancer tissues and B2 (Fig. 6B). A similar expression pattern was observed for AR, which, as expected, is expressed higher in C1 and C2 tissues than in the other tissues (Fig. 6B). Together, these limited tissue expression data show a similar expression pattern for ETV1, c-Jun, and AR.

**Discussion**

Several earlier studies from our lab have characterized the coactivation function of c-Jun on AR transcriptional activity.
These studies identified regions of c-Jun necessary for AR coactivation (6) and regions of AR that respond to this c-Jun activity (27); showed that heterodimerization with c-Fos antagonizes c-Jun coactivation (5); functionally separated c-Jun coactivation from transactivation (6); and importantly suggested that c-Jun coactivation supports prostate cancer cell proliferation whereas c-Jun transactivation is antiproliferative (19). In contrast to these two opposite effects of c-Jun on proliferation, our study here provides evidence that both the c-Jun coactivation and transactivation functions have a positive effect on prostate cancer cell invasion. In addition, this proinvasive effect of c-Jun in prostate cancer seems to depend on multiple activities by this proto-oncoprotein in a novel pathway that links the AR to ETV1 and is described in another article. In that article, we reported that ETV1 is transcriptionally induced by ligand-activated AR in prostate cancer cells. In the current study, we describe c-Jun activities in the entire pathway that begins with AR transactivation of ETV1 and terminates with prostate cancer cell invasion (see Fig. 6A). Thus, the role of c-Jun in prostate cancer cell invasion seems to be mediated by multiple activities of this proto-oncoprotein.

c-Jun acts in the beginning of the AR-ETV1 invasion pathway by enhancing ETV1 expression in prostate cancer cells. Our data suggest that this enhancement is due to (a) c-Jun coactivation of AR and (a) c-Jun direct transactivation of the ETV1 promoter. The coactivation function is shown by our finding that either transient (see Fig. 1F) or stable expression, as in M37 cells (see Fig. 1G), of the transactivation-deficient mutant c-Jun(Ala63/73) led to elevated expression of ETV1. This c-Jun mutant has been shown to be unable to activate AP-1–regulated promoters (6) and, in this study, the cloned ETV1 promoter that responds to ligand-activated AR. By contrast, wild-type c-Jun exhibited strong activity on the ETV1 promoter (see Fig. 1F), showing that c-Jun can transactivate the ETV1 gene independent of ligand-activated AR. Whereas overexpression of c-Jun led to elevated ETV1 expression, siRNA-mediated diminution of c-Jun resulted in substantially reduced ETV1 expression (see Fig. 1E), strongly suggesting that endogenous c-Jun and/or stably expressed c-Jun(Ala63/73) is involved in ETV1 expression in M37 cells. However, because siRNA inhibits the expression of both wild-type and mutant c-Jun, it is impossible to determine from this experiment which of the two c-Jun activities, transactivation or coactivation, is more important for ETV1 expression in prostate cancer cells.

Not only is c-Jun able to elevate ETV1 expression but our data show that it can also mediate ETV1 transcriptional activity, as measured by both reporter gene assays and endogenous gene expression. With respect to the reporter gene assays, both the Fes and MMP-1 promoters are about equally transactivated by either exogenous ETV1 or wild-type c-Jun (see Fig. 3A and B). Interestingly, transfected c-Jun(Ala63/73) had weaker but significant positive activity on both promoters (see Fig. 3A and B), probably due to its ability to cooperate with endogenous ETV1 to activate transcription. This possibility is supported by our finding that the Fes promoter had higher activity in M37 cells than in LNCaP cells both in the presence and absence of DHT (see Fig. 3F); the increased activity without DHT probably reflects the ability of the c-Jun(Ala63/73) protein expressed in M37 cells to enhance endogenous ETV1 activity on the Fes promoter. The effect of coexpressed ETV1 and c-Jun was strongly synergistic on the Fes promoter and weakly on the MMP-1 promoter (see Fig. 3A and B), perhaps reflecting a promoter-specific difference in cooperation magnitude between ETV1 and c-Jun. The cooperative activity
of c-Jun on ETV1 transactivation does not depend on c-Jun DNA binding, as shown by the equal ability of a DNA binding-deficient mutant as wild-type c-Jun to support ETV1 activation of the MMP-1 promoter (see Fig. 3D).

Interestingly, c-Jun enhancing activity has previously been reported on several Ets transcription factors, including PU.1 (26) and all three PEA3 group proteins (12, 22). Here, we have provided evidence that this c-Jun enhancing activity also occurs on MMP gene expression. Our earlier work suggested that MMP-9 and MMP-13, like MMP-1 (27) and MMP-7 (12), are ETV1 target genes. In this study, we show that MMP gene expression is higher in M37 cells than in control LNCaP cells (see Fig. 4B). Similar results to those from M37 cells were obtained when c-Jun(Ala63,73) was transiently expressed in LNCaP cells (see Fig. 4A), showing that overexpression of the transactivation-deficient mutant was sufficient to enhance MMP gene expression. All these results show that the transactivation function of c-Jun is not necessary for enhanced ETV1 transcriptional activity on MMP gene expression and thus suggest that c-Jun may act as a coactivator for ETV1, as it does for AR. This coactivation function is likely mediated by a physical interaction between c-Jun and AR or ETV1 in LNCaP cells, as our immunoprecipitation and immunocytochemistry data here suggest (see Fig. 5A and B). Interestingly, the immunocytochemistry data also suggest that c-Jun/AR nuclear colocalization sites may be different from the c-Jun/ETV1 sites, and thus suggest that c-Jun cooperates with AR or ETV1 by associating with these two proteins at distinct nuclear sites. To obtain evidence for this, we did chromatin immunoprecipitation assays showing that c-Jun can be recruited in an androgen-dependent manner to the ETV1 promoter region (see Fig. 2E) that also recruits ligand-activated AR. Importantly, c-Jun is also recruited to the PSA promoter but not the sGco1 promoter (see Fig. 2E). In view of our data showing that c-Jun(Ala63,73) can enhance the expression of ETV1 (see Fig. 1G) and PSA (19) but not sGco12 (see Table 1), our chromatin immunoprecipitation data here strongly suggest that c-Jun is recruited in an androgen-induced manner only to those promoters that respond to c-Jun coactivation. Additionally, these data suggest that the c-Jun physical association with AR (see Fig. 2A-D) is responsible for a promoter-specific recruitment of c-Jun, providing the first evidence that ligand-activated AR can bring c-Jun to AR-regulated promoters.

Just as c-Jun enhancing activity on ETV1 expression seems to be mediated by its coactivation and transactivation functions, so too is the effect of c-Jun on MMP gene expression. It was reported several years ago that c-Jun directly activates MMP genes through its transactivation function as a component of AP-1 (28). Thus, taking into account our data presented here together with published results produces a complex and interesting model for c-Jun action in prostate cancer. c-Jun has multiple transcriptional functions as a coactivator and transactivator, which can lead to increased expression and activity of ETV1, resulting in elevated expression of MMPs or other functionally related gene(s) that can then induce prostate cancer cell invasion (Fig. 6C). In addition, our previous data suggest that c-Jun coactivation can also enhance prostate cancer cell proliferation, whereas c-Jun transactivation, surprisingly, is antagonistic to proliferation (19). These multiple effects of c-Jun on prostate cancer behavior are undoubtedly due to differential gene expression that is induced by c-Jun coactivation of AR and ETV1 and direct transactivation as a part of AP-1. As an initial analysis of the c-Jun coactivation function on androgen-regulated genes, a gene microarray system was used to measure expression of androgen-regulated genes in LNCaP cells expressing c-Jun(Ala63,73), a mutant deficient in transactivation but fully competent in AR coactivation (6). This analysis shows that some androgen-regulated genes, including ETV1, exhibit elevated expression induced by c-Jun coactivation, whereas other genes are not changed in their expression (see Table 1). This finding clearly shows that c-Jun coactivation of AR is not general but target gene specific. Perhaps c-Jun also has gene-specific effects on ETV1 transcriptional activity, a hypothesis that will form the basis of future studies aimed at identifying genes mediating the ETV1 effect on prostate cancer cell invasion.

Materials and Methods

Cell Culture and siRNA Transfection

The culturing of LNCaP, C33, C81, C14, M37, AJ81, and A103 cells has been described (19). Note that the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin was similar among these different cell lines, showing that housekeeping gene expression is not altered in these cells. Commercial siRNAs were obtained for ETV1 (Ambion), c-Jun, and negative control (both from Santa Cruz Biotechnology). Note that we used two different siRNAs targeting different regions of ETV1 or c-Jun, and these had similar individual effects. siRNA was transfected into cells using X-tremeGENE siRNA transfection following the manufacturer’s protocol (Roche). Plasmid DNA was transfected using Lipofectamine 2000 following the manufacturer’s protocol (Invitrogen).

Cells were grown to 60% to 70% confluency in 10% fetal bovine serum (FBS)—containing medium and then changed to serum-free medium. After 48 h of incubation, ethanol or 100 mmol/L DHT was added to the cells. After an additional 48 h of incubation, the cells were subjected to either semiquantitative RT-PCR or Western blotting.

Plasmid and Reporter Gene Assay

The cloning of the human ETV1 promoter to make the reporter plasmid ETV1-Luc has previously been described. This promoter exhibits androgen-induced activation and contains a near consensus androgen-responsive element that is able to recruit liganded AR in a chromatin immunoprecipitation assay. The other reporter plasmids used, MMP1-Luc (23), MMP1(mAP1)-Luc (25, 29), and Fes3×WT-Luc (pBfes.Luc; ref. 20), have previously been described.

For reporter gene assays, cells were transfected and luciferase assays done as previously described (19). For all transfections, empty vector (empty expression plasmid or promoter-less reporter plasmid) was used to ensure equal amounts of each kind of vector, and pCH110, which expresses β-galactosidase, was used to standardize transfection efficiency (16). Note that the β-galactosidase activity was very similar in all the prostate cancer cell lines used, showing that the
constitutive promoter driving the expression of β-galactosidase was equally active among the different cell lines. All luciferase values represent the average of three independent transfections plus standard deviations.

Semiquantitative RT-PCR and Real-time Quantitative PCR Analyses

RNA was isolated using the TRIZol reagent and subjected to either semiquantitative RT-PCR as previously described (19) or real-time quantitative-PCR using SYBR Green (iSCRIPT from Bio-Rad). The upstream and downstream primers, respectively, used for each gene were PSA, 5'-GCAGCATGAAAAGGAGGAGG-3' and 5'-CCCAGTCGATGACCCCTTTA-3'; c-Jun, 5'-TGACTGGAAGATGAAAGC-3' and 5'-CCGTTCGCTGGACTGGATT-3'; AR, 5'-CAATGAGTACCGCATG-3' and 5'-GCCCATCCACTGGAATAATG-3'; ETV1, 5'-TACCCCATGGACCACAGATT-3' and 5'-CAGCTGGACTCATTGTCG-3'; MMP-7, 5'-GAGGTGCCAGATGTTGCAAA-3' and 5'-AAATTGCAAGGGGATCTCTTT-3'; MMP-9, 5'-GCCATTCACTGCTGCTTTAT-3' and 5'-TTGGACAGCAGGAAAGTG-3'; MMP-13, 5'-GAGGCTCTCAGTGATCATG-3' and 5'-TTGAGCTGAGACTTCTTGC-3'; and GAPDH, 5'-CGACCACCTTTGCAAGCTCA-3' and 5'-GCCCATCCACTGGAATAATG-3'. GAPDH was used as a control for mRNA amount.

Immunoprecipitation

Whole-cell extracts from LNCaP cells grown to 90% confluence in 10% FBS containing RPMI 1640 were subjected to immunoprecipitation following the protocol from Upstate, using protein A-Sepharose (Amersham). The anti-ETV1 antibody NTD (15) and anti-c-Jun antibody sc-45 (Santa Cruz Biotechnology) were used to immunoprecipitate ETV1 or c-Jun, respectively. The anti-AR antibody PA1-110 (Affinity Bioreagents) and anti–c-Jun antibody sc-45 (Santa Cruz Biotechnology) were used to immunoprecipitate following the protocol from Upstate, using protein A-Sepharose (Amersham). The anti-ETV1 antibody NTD (15) and anti-c-Jun antibody sc-45 (Santa Cruz Biotechnology) were used for immunoprecipitation.

Affymetrix Gene Chip Assay

C14 and M37 cells were grown to 60% to 70% confluence in 10% FBS and then changed to FBS-free medium. After 24 h of incubation, cells were treated with either ethanol or 100 nmol/L DHT. After 48 h of incubation, total mRNA was isolated and subjected to gene chip analysis using chips purchased from Affymetrix (GeneChip Human Genome U95Av2 Array) according to the manufacturer’s protocol.

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References


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