IFIT16 in Human Prostate Cancer

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Abstract
Increased expression of IFIT16 protein (encoded by the IFIT16 gene) in normal human prostate epithelial cells is associated with cellular senescence-associated cell growth arrest. Consistent with a role for IFIT16 protein in cellular senescence, the expression of IFIT16 protein is either very low or not detectable in human prostate cancer cell lines. We now report that treatment of DU-145 and LNCaP prostate cancer cell lines with histone deacetylase inhibitor trichostatin A (TSA) or CGK1026 resulted in transcriptional activation of the IFIT16 gene. The induction of IFIT16 protein in LNCaP cells was dependent on the duration of TSA treatment. Furthermore, TSA treatment of LNCaP cells up-regulated the expression of Janus-activated kinase 1 protein kinase and modulated the transcription of certain IFN-activatable genes. However, overexpression of exogenous Janus-activated kinase 1 protein in LNCaP cells and treatment of cells with IFNs (α and γ) did not increase the expression of IFIT16. Instead, the transcriptional activation of IFIT16 gene by TSA treatment of LNCaP cells was dependent on transcriptional activation by c-Jun/activator protein-1 transcription factor. Importantly, increased expression of IFIT16 in LNCaP cells was associated with decreases in the expression of androgen receptor and apoptosis of cells. Conversely, knockdown of IFIT16 expression in TSA-treated LNCaP cells increased androgen receptor protein levels with concomitant decreases in apoptosis. Together, our observations provide support for the idea that histone deacetylase–dependent transcriptional silencing of the IFIT16 gene in prostate epithelial cells contributes to the development of prostate cancer.

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Introduction
IFNs, a family of cytokines, are potent inhibitor of cell growth and also exhibit antitumor activity (1, 2). The family includes type-I (IFN-α and IFN-β) and type-II (IFN-γ) IFNs, among others. The Janus family tyrosine kinase that is used by both IFN-γ and IFN-α/β receptors to activate signal transducer and activator of transcription (STAT) proteins is Janus-activated kinase 1 (JAK1; ref. 1). Transcriptional activation of IFN-activatable genes by activated STATs results in induction of IFN-inducible proteins that mediate the growth-inhibitory activities of IFNs. Importantly, studies have revealed that defects in IFN signaling, which result in the lack of expression of IFN-inducible proteins, are associated with immortalization of human cells (3) and development of certain cancers, including the prostate cancer (4, 5).

Histone acetylation or deacetylation represents the best-studied posttranslational modification of core histones and is a pivotal mechanism for the control of gene transcription (6, 7). Two opposing types of enzymes, the histone acetyltransferases and the histone deacetylases (HDACs), regulate the acetylation status of nucleosomal core histones and other substrates. Transcriptional activators usually possess histone acetyltransferase activity, whereas repressors interact with HDACs, whose activity is linked to silencing of gene transcription. Importantly, several studies have provided support for the idea that transcriptional silencing of the growth/tumor suppressor genes by HDACs contributes to the development of human cancers (8). Intriguingly, several studies have suggested that the HDAC activity is needed for transcriptional activation/modulation of certain IFN-activatable genes (9, 10).

Studies have indicated that treatment of human prostate cancer cell lines with inhibitors of HDACs suppresses cell growth (11, 12). Moreover, studies have indicated that inhibitors of HDACs differentially induce apoptosis in prostate cancer cells (13, 14). Importantly, treatment of LNCaP cells with trichostatin A (TSA) is shown to induce cell death through down-regulation of the transcription of the androgen receptor (AR) gene (14). However, the molecular mechanisms remain unknown.

A study has reported that human LNCaP prostate cancer cell line is defective in IFN signaling because cells do not express the JAK1 tyrosine kinase (15). The study also showed that treatment of LNCaP cells with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase, and TSA, an inhibitor of HDACs, results in up-regulation of JAK1 mRNA. However, it remains unknown whether the expression of JAK1 in LNCaP cells is sufficient to induce the expression of IFN-inducible proteins that mediate the growth-inhibitory functions of IFNs.

Although treatment of human breast cancer cell lines with 5-aza-dC has been reported to induce the expression of IFIT16 gene (16), the presence of a CpG island has not been shown in the 5′-regulatory region of IFIT16 gene. Consistent with a role for IFIT16 protein in cellular senescence-associated cell growth arrest, we have noted (5) that the expression of IFIT16 protein was either very low or undetectable in most human prostate cancer cell lines tested (including LNCaP and DU-145 cell lines).
Importantly, we found that overexpression of IFI16 protein in LNCaP and DU-145 prostate cancer cell lines results in inhibition of cell growth (5). Furthermore, we have reported that increased expression of IFI16 protein in LNCaP prostate cancer cells down-regulates the AR expression and inhibits AR-mediated functions (17).

In the present study, we have investigated the molecular mechanisms by which the expression of IFI16 gene is impaired in human prostate cancer cell lines. Additionally, we have examined the molecular mechanisms by which increased expression of IFI16 in LNCaP prostate cancer cells down-regulates the AR expression and inhibits AR-mediated functions (17).

In the present study, we have investigated the molecular mechanisms by which the expression of IFI16 gene is impaired in human prostate cancer cell lines. Additionally, we have examined the molecular mechanisms by which increased expression of IFI16 in LNCaP prostate cancer cells inhibits cell growth. We report that treatment of LNCaP and DU-145 prostate cancer cell lines with inhibitors of HDACs, such as TSA or CGK1026, resulted in up-regulation of IFI16 expression. Additionally, we found that increased expression of IFI16 protein in LNCaP cells was associated with down-regulation of AR expression and an increase in apoptosis of cells.

**Results**

Treatment of Prostate Cancer Cell Lines with Inhibitors of HDACs Up-Regulates the Expression of IFI16

We have reported previously that expression of IFI16 protein is not detectable in androgen-responsive LNCaP and androgen nonresponsive DU-145 prostate cancer cell lines (5). Because treatment of immortalized cells with 5-aza-dC, an inhibitor of DNA methyltransferase, results in up-regulation of IFI16 (18), we explored whether treatment of LNCaP cells with 5-aza-dC and/or TSA, an inhibitor of HDACs, has any effect on the expression of IFI16. For this purpose, we treated LNCaP cells with 5-aza-dC, TSA, or both and analyzed the expression of IFI16 protein by immunoblotting. For immunoblotting, we used optimized conditions (19) that allowed us the detection of relatively high molecular weight proteins, such as IFI16, at relatively low levels.

**FIGURE 1.** Treatment of human prostate cancer cell lines with HDAC inhibitors results in up-regulation of IFI16 expression. A. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with TSA (100 nmol/L; lane 2) for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. B. Total RNA isolated from control (lane 1) or TSA-treated (100 nmol/L for 24 h; lane 2) LNCaP cells were subjected to RT-PCR, using a set of primers (specific to IFI16 gene or β-actin gene) as described in Materials and Methods. C. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with the indicated concentrations of TSA (lanes 2-4) for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. D. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with 100 nmol/L TSA for the indicated duration (h) were analyzed by immunoblotting using antibodies specific to the indicated proteins. E. Total cell extracts prepared from control (lane 1) DU-145 cells or cells treated with IFNs (IFN-α, 1,000 units/mL; IFN-γ, 20 ng/mL; lane 2), TSA (300 nmol/L; lane 3), or IFNs and TSA together for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. F. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with the indicated concentrations of CGK1026 (lanes 2 and 3) were analyzed by immunoblotting using antibodies specific to the indicated proteins.
(25 nmol/L) concentrations of TSA (Fig. 1C). Furthermore, the induction of IFI16 protein was dependent on the duration of the treatment: the maximal induction was evident after 16 h of TSA treatment of cells (Fig. 1D). Similarly, treatment of DU-145 cells with TSA also resulted in up-regulation of IFI16 protein (Fig. 1E) albeit moderately.

Because TSA treatment of cells is known to inhibit the activity of HDACs differentially (13), we also treated LNCaP cells with CGK1026, a recently identified inhibitor of HDACs (20). As shown in Fig. 1F, treatment of LNCaP cells with CGK1026 also resulted in up-regulation of IFI16 protein in a dose-dependent manner. Together, these observations provide support for the idea that the expression of IFI16 gene in LNCaP and in DU-145 prostate cancer cells is impaired by HDACs.

TSA-Mediated Increased Expression of IFI16 in LNCaP Cells Is Not Dependent on Increased Expression of JAK1 Kinase

It has been reported that treatment of LNCaP cells with both TSA and 5-aza-dC activates the transcription of JAK1 gene that encodes a tyrosine kinase, which is needed for the activation of STATs (STAT1 and STAT2) after IFN-α/β and IFN-γ treatment of cells (15). Therefore, we tested whether treatment of LNCaP cells with 5-aza-dC, TSA, or both has any effect on JAK1 expression. In contrast to the previous report (15), we could detect basal low levels of JAK1 protein in LNCaP cells by immunoblotting (Fig. 3A), and the treatment of cells with TSA, but not 5-aza-dC, resulted in increases in levels of the JAK1 protein kinase (Fig. 3A, compare lane 1 with lane 3). Furthermore, treatment of cells with both TSA and 5-aza-dC together did not result in further increases in JAK1 protein levels.

Increased levels of JAK1 protein in LNCaP cells after TSA treatment and TSA-mediated increases in the steady-state levels of mRNAs encoding the IFN-inducible p27 and p35 proteins raised the possibility that the increased expression of JAK1 in LNCaP cells contributes to the transcriptional activation of the IFI16 gene. To test this possibility, we nucleofected the LNCaP cells with an empty vector or the plasmid encoding human JAK1 protein. To activate JAK1 kinase, we treated cells with IFNs (α and γ) or, as a control, left cells untreated and analyzed the expression of IFI16 protein. As shown in Fig. 3B, nucleofection of cells with the plasmid encoding JAK1 resulted in increased expression of JAK1 protein. However, treatment of LNCaP cells that overexpressed JAK1 protein kinase with IFNs (α and γ) did not result in induction of IFI16 protein. Similarly, no increase was evident in levels of OAS protein, another well-known IFN-inducible protein (21).

Because the Tyr701 phosphorylation of STAT1 protein by JAK1 tyrosine kinase activates the STAT1 protein (1), resulting in transcriptional activation of the target genes, the above observations prompted us to test whether TSA-mediated up-regulation of JAK1 protein in LNCaP cells results in Tyr701 phosphorylation of STAT1 protein. As shown in Fig. 3C, treatment of LNCaP cells with TSA resulted in up-regulation of JAK1 protein. However, treatment of cells with TSA and IFNs (α and γ) did not result in further increases in JAK1 levels (Fig. 3C, compare lane 3 with lane 4). Furthermore, neither TSA treatment of LNCaP cells nor the treatment with both TSA and IFNs (α and γ) resulted in Tyr701 phosphorylation of STAT1 protein, indicating that TSA-mediated up-regulation of IFI16 protein in LNCaP cells does not depend on transcriptional activation by STAT1 protein.
the IFI16 gene (25), we explored whether TSA treatment of LNCaP cells stimulates the DNA-binding activity of AP-1 transcription factor. Consistent with the previous report (24), we were unable to detect any specific DNA-binding activity of proteins from control LNCaP cells to an oligonucleotide containing the AP-1 DNA-binding consensus sequence in gel mobility shift assays (Fig. 4A). Of note, TSA treatment of LNCaP cells resulted in increased specific DNA-binding activity of proteins to the AP-1 oligonucleotide. Moreover, incubation of the extracts with antibodies to c-Jun or an isotype antibody followed by gel mobility shift assays revealed that the incubation of extracts with antibodies to c-Jun, but not an isotype antibody, under our experimental conditions resulted in decreases in binding of protein complexes to the AP-1 consensus sequence (Fig. 4B, left). This observation indicated that the binding of the antibody to c-Jun in protein complexes inhibited the binding of protein complex to the AP-1 oligonucleotide. Moreover, in extracts from TSA-treated LNCaP cells, we also detected increased specific DNA binding of proteins to an oligonucleotide containing the c-Jun/AP-1 DNA-binding consensus sequence found in the IFI16 gene (Fig. 4B, right; see ref. 26). Importantly, consistent with TSA-stimulated increased specific DNA-binding activity of c-Jun/AP-1 in gel mobility shift assays, treatment of LNCaP cells with TSA also resulted in 6-fold stimulation of the activity of AP-1–luc reporter (Fig. 4C). Similarly, the activity of IFI16-luc reporter was also stimulated ~6-fold in response to TSA treatment.

Because phosphorylation of c-Jun by c-Jun NH2-terminal kinase (JNK) activates c-Jun/AP-1–mediated transcription (27), we tested whether TSA treatment of LNCaP cells results in the activating phosphorylation of c-Jun. As shown in Fig. 4D, we found that treatment of LNCaP cells with TSA resulted in phosphorylation of c-Jun and up-regulation of c-Jun protein. Furthermore, the treatment of cells with TSA and IFNs (α and γ) did not have any effect on phosphorylation of c-Jun and c-Jun protein levels (Fig. 4D, compare lane 3 with lane 4). Importantly, the phosphorylation and activation of c-Jun in LNCaP cells was correlated with up-regulation of IFI16 protein. Consistent with the above observations, treatment of LNCaP cells with anisomycin, a potent activator of c-Jun/AP-1 activity in cells (28), also resulted in up-regulation of IFI16 protein (Fig. 4E). Moreover, treatment of LNCaP cells with a JNK inhibitor (SP600125) reduced the extent of TSA-mediated up-regulation of IFI16 protein (Fig. 4F, compare lane 2 with lane 4). Together, these observations indicated that TSA-mediated up-regulation of IFI16 in LNCaP cells depends on transcriptional activation of genes by c-Jun/AP-1.

Up-Regulation of IFI16 in LNCaP Cells Down-Regulates AR Expression

Our previous studies had revealed that overexpression of IFI16 in DU-145 and LNCaP cells inhibits cell growth in colony formation assays (5). Additionally, we have noted (17) that overexpression of IFI16 in LNCaP cells down-regulates AR expression, inhibits the AR-mediated transcription of target genes, and up-regulates the expression of cell growth–inhibitory proteins. Therefore, TSA-mediated up-regulation of IFI16 in LNCaP cells prompted us to test whether increased expression of IFI16 is associated with decreases in AR expression and inhibition of cell growth. Consistent with our previous observations (17), we found that TSA-mediated increased expression of IFI16 protein in LNCaP was associated

![FIGURE 3. TSA-mediated increased expression of IFI16 in LNCaP cells is not dependent on increased expression of JAK1 protein kinase. A. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with 5-aza-dC (5-aza; 5 μmol/L; lane 2), TSA (100 nmol/L; lane 3), or both 5-aza-dC and TSA together (lane 4) for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. The treatment-induced fold changes in JAK1 protein levels are at the bottom of the figure. B. LNCaP cells were either nucleofected with an empty vector (lanes 1 and 3) or a plasmid encoding human JAK1 protein kinase (lanes 2 and 4) as described in Materials and Methods. Twenty-four hours after nucleofections, cells were either left untreated (lanes 1 and 2) or treated with IFNs (IFN-α, 1,000 units/mL; IFN-γ, 20 ng/mL; lanes 3 and 4). Forty-eight hours after nucleofections, total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins. C. Total cell extracts prepared from control (lane 1) LNCaP cells or cells treated with IFNs (IFN-α, 1,000 units/mL; IFN-γ, 20 ng/mL; lane 2), TSA (300 nmol/L; lane 3), or IFNs and TSA together for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins.](mcr.aacrjournals.org)
with ~50% decreases in the steady-state levels of AR mRNA (Fig. 5A) and protein (Fig. 5B). Similarly, TSA-mediated increases in IFI16 protein levels in DU-145 cells (Fig. 1E) were associated with decreases in AR protein levels (data not shown). Because treatment of LNCaP cells with HDAC inhibitor LAQ824 has been shown to down-regulate AR protein levels by reducing the expression of heat shock protein 90 (HSP90) protein (29), we also tested whether TSA treatment

![Diagram of experimental setup](image_url)

**FIGURE 4.** TSA-mediated up-regulation of IFI16 expression in LNCaP cells depends on activation of c-Jun/AP-1 transcription factor. A. Samples containing equal amounts of protein from control (lanes 1 and 3) or TSA-treated (lanes 2 and 4) LNCaP cells were either incubated with radiolabeled AP-1 oligonucleotide (1 ng; probe) alone (lanes 1 and 2) or along with 50-fold molar excess of cold AP-1 oligonucleotide. Samples were subjected to gel mobility shift assays as described in Materials and Methods. Arrow, the AP-1–specific gel mobility shift band. B. Left, samples containing equal amounts of protein from control (lanes 1 and 3) or TSA-treated (lanes 2 and 4) LNCaP cells were either incubated with an isotype antibody (2 μg) or antibody to c-Jun (2 μg) for 30 min on ice. These samples were subjected to gel mobility shift assays using the radiolabeled AP-1 oligonucleotide (1 ng) as described in Materials and Methods. Arrow, the AP-1–specific gel mobility shift band. Right, samples containing equal amounts of protein from control (lanes 2, 4, and 6) or TSA-treated (lanes 3, 5, and 7) LNCaP cells were either incubated with radiolabeled oligonucleotide (1 ng; probe) containing the AP-1 binding consensus sequence from IFI16 gene promoter (IFI16 oligonucleotide) alone (lanes 2 and 3), along with 50-fold molar excess of cold AP-1 oligonucleotide (lanes 4 and 5), or IFI16 oligonucleotide (lanes 6 and 7). As a control, the probe without any extracts (lane 1) was also incubated. Samples were subjected to gel mobility shift assays as described in Materials and Methods. Arrow, the AP-1–specific gel mobility shift band. NS, nonspecific bands. C. LNCaP cells were nucleofected with either AP-1–luc reporter plasmid (1.8 μg) or IFI16-luc reporter plasmid (1.8 μg) along with pRL-TK reporter plasmid (0.2 μg) as described in Materials and Methods. Twenty-four hours after nucleofections, cells were either left untreated (control) or treated with TSA (100 nm). Forty-five hours after nucleofections, cells were processed for dual-luciferase reporter activity assays as described in Materials and Methods. The firefly luciferase reporter activity was normalized to the Renilla luciferase activity to control for variations in nucleofection efficiencies. 1, the luciferase activity in control cells. D. Extracts prepared from LNCaP cells either left untreated (control; lane 1) or treated with IFNs (IFN-α, 1,000 units/mL; IFN-γ, 20 ng/mL) alone (lane 2), TSA (100 nm) alone (lane 3), or TSA and IFNs for 24 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. E. Extracts prepared from LNCaP cells either left untreated (control; lane 1) or treated with anisomycin (20 μM) for 4 h were analyzed by immunoblotting using antibodies specific to the indicated proteins. F. Extracts prepared from LNCaP cells either left untreated (control; lane 1) or treated with TSA (100 nm) alone (lane 2), JNK inhibitor SP600125 (20 μM) alone (lane 3), or TSA and JNK inhibitor for 22 h were analyzed by immunoblotting using antibodies specific to the indicated proteins.
of LNCaP cells has any effect on HSP90 protein levels. As shown in Fig. 5B, treatment of cells with TSA did not have any effect on HSP90 protein levels, indicating that TSA-mediated down-regulation of AR protein in LNCaP cells is independent of HSP90 protein levels. However, it remains a possibility that TSA-mediated down-regulation of AR protein in LNCaP cells depends on acetylation of HSP90 protein, which could affect its chaperone function.

Because the expression of AR protein is needed for survival of LNCaP cells (26, 30), a reduction in AR protein levels after treatment of LNCaP cells with TSA prompted us to test whether the reduction in AR protein levels was associated with apoptosis of cells. As shown in Fig. 5B, treatment of cells with TSA resulted in increased cleavage of poly(ADP-ribose) polymerase (PARP) protein, which is indicative of caspase-mediated apoptosis of cells (31). Consistent with these observations, fluorescence-activated cell sorting analyses of propidium iodide–stained control and TSA-treated LNCaP cells, which resulted in up-regulation of IFI16 protein and down-regulation of AR protein, revealed that TSA treatment significantly increased apoptosis of cells (Fig. 5C). Because increased expression of IFI16 protein in LNCaP cells negatively regulates the expression of AR gene (17) and down-regulation of AR protein in LNCaP cells is known to result in apoptosis of cells (26, 30), we tested whether knockdown of IFI16 expression in TSA-treated LNCaP cells has any effect on AR protein levels and the extent of apoptosis of cells. As shown in Fig. 5D, consistent with our previous observations (18), nucleofection with small interfering RNA (siRNA) IFI16, but not siRNA control, resulted in >50% knockdown of IFI16 protein in TSA-treated LNCaP cells. Importantly, this reduced expression of IFI16 protein was associated with...
~ 50% increases in the AR protein levels and a decrease in the extent of the cleavage of PARP protein (Fig. 5D, compare lane 1 with lane 2). Together, these observations indicated that TSA-induced increased expression of IFI16 in LNCaP cells inhibits cell growth, in part, by down-regulating the expression of AR and increasing the susceptibility to apoptosis.

Discussion

The 200-gene family of IFN-activatable genes includes mouse (e.g., Ifi202a, Ifi202b, Ifi203, and Ifi204) and human genes (e.g., IFI16, MNDA, and AIM2) that encode structurally related proteins (the p200-family proteins; ref. 32). Increased expression of the p200-family proteins, such as p202 and IFI16, is known to inhibit cell cycle progression and modulate apoptosis (33). Moreover, the p200-family proteins function as scaffold proteins and their binding to transcription factors is known to modulate the transcription of genes.

We have reported (5) that normal human prostate epithelial cells express all three forms (A, B, and C) of IFI16 protein. Furthermore, we have noted that increased expression of IFI16 protein in old (versus young) populations of normal human diploid fibroblasts (21) and in normal human prostate epithelial cells (5) is associated with cellular senescence-associated cell growth arrest, and immortalization of normal human cells is associated with down-regulation of IFI16 mRNA and protein (18). Moreover, consistent with a role for IFI16 protein as a scaffold protein, IFI16 protein binds to p53 (34), pRb and E2F1 (5), and BRCA1 (35) proteins. Binding of IFI16 to Rb and E2F1 is correlated with inhibition of E2F1-mediated transcription of target genes (5, 17).

We have reported (5) that the expression of IFI16 protein is not detectable in certain human prostate cancer cell lines (e.g., LNCaP and DU-145). Interestingly, a study found that the expression of IFI16 protein is relatively low in human breast cancer cell lines and tumors (compared with normal breast epithelial cells and tissues). Furthermore, treatment of these breast cancer cell lines with 5-aza-dC resulted in up-regulation of IFI16 expression (16). Consequently, we considered the possibility that either our immunoblotting conditions to detect relatively low levels of IFI16 protein in prostate cancer cell lines were not optimum or the expression of IFI16 gene is silenced in prostate cancer cell lines. Therefore, we chose to use our recently optimized (19) immunoblotting conditions to detect the expression of IFI16 protein. Using the optimized conditions for immunoblotting, we were able to detect basal low levels of IFI16 protein in both LNCaP and DU-145 cells (Fig. 1). Moreover, we found that treatment of LNCaP and DU-145 cells with HDAC inhibitors (TSA or CGK1026) resulted in up-regulation of IFI16 mRNA and protein (Fig. 1). To our knowledge, this is the first report providing evidence that HDACs negatively regulate the expression of IFI16 gene in human prostate cancer cell lines.

Increased expression of HDACs has been implicated in the development and progression of prostate cancer (36). Moreover, HDAC inhibitors have shown significant antiproliferative and apoptotic properties on various cancer cells, including the prostate cancer cells (13). Therefore, our observations (a) that treatment of prostate cancer cell lines (LNCaP and DU-145) with TSA (or CGK1026) resulted in up-regulation of IFI16 mRNA and protein (Fig. 1); (b) that increased expression of IFI16 in LNCaP cells was associated with down-regulation of AR expression and apoptosis of cells (Fig. 5); and (c) that knockdown of IFI16 expression in TSA-treated LNCaP cells resulted in up-regulation of AR and inhibition of PARP cleavage (Fig. 5) provide support for the idea that HDAC-mediated decreased expression of IFI16 gene in prostate epithelial cells contributes to up-regulation of AR expression and survival of cells.

Recent findings have indicated that positive regulation of gene expression by IFNs and other cytokines requires both acetylation and deacetylation (10). Consistent with this idea, a recent study reported that treatment of LNCaP cells with both 5-aza-dC and TSA activates the transcription of JAK1 gene, resulting in detection of JAK1 mRNA by RT-PCR and activating phosphorylation of STATs by IFNs in JAK1-transfected cells (15). However, in contrast to the above report, we were able to detect (Fig. 3) the basal levels of JAK1 protein in LNCaP cells under our optimized immunoblotting conditions, and treatment of cells with TSA, but not 5-aza-dC, resulted in up-regulation of JAK1 protein. Moreover, the treatment of cells with TSA resulted in differential regulation of the steady-state levels of mRNAs for certain IFN-activatable genes (Fig. 2). Intriguingly, treatment of LNCaP cells that overexpressed JAK1 protein with IFNs (α and γ) did not result in up-regulation of IFI16 protein or another IFN-inducible protein OAS. Furthermore, treatment of LNCaP cells with both TSA and IFNs did not result in activating phosphorylation of STAT1 protein. Together, these observations showed that up-regulation of JAK1 protein by TSA treatment of LNCaP cells is not sufficient to induce the expression of certain IFN-inducible proteins, such as IFI16 and OAS.

A study has suggested a critical role for the transcription factor c-Jun/AP-1 in the constitutive and IFN-induced expression of the IFI16 gene (25). Because inhibition of HDAC activity by TSA is known to activate AP-1–mediated transcription (22, 23), our observations that TSA treatment of LNCaP cells resulted (α) in stimulation of the specific DNA-binding activity of AP-1 transcription factor in gel mobility shift assays (Fig. 4); (b) in ~ 6-fold stimulation of the activity of both AP-1– and IFI16-luc reporters (Fig. 4); and (c) in activating phosphorylation of c-Jun and its autostimulation of expression (Fig. 4) provide support for the idea that TSA-mediated transcriptional activation by c-Jun/AP-1 factor contributes to the transcriptional activation of IFI16 gene in LNCaP cells. Consistent with this idea, we also noted that treatment of LNCaP cells with anisomycin, an activator of AP-1 (28), also resulted in activating phosphorylation of c-Jun and up-regulation of IFI16 protein (Fig. 4). Moreover, treatment of cells with TSA and an inhibitor of JNK (JNK phosphorylates c-Jun to activate its activity; see ref. 27) resulted in inhibition of phosphorylation of c-Jun and concomitant inhibition in increases in expression levels of IFI16 protein (Fig. 4). Together, these observations suggested that TSA treatment of LNCaP cells activates the transcription of IFI16 gene in JNK/c-Jun/AP-1–dependent manner.

We have reported (18) that treatment of immortalized human fibroblasts with 5-aza-dC, which results in demethylation of
genomic DNA, results in up-regulation of IFI16 protein. However, the presence of a CpG island could not be shown in the promoter region of the IFI16 gene (18), raising the possibility that DNA methylation indirectly regulates the expression of IFI16 gene.

Our observations described here that treatment of prostate cancer cell lines (LNCaP and DU-145) with inhibitors of HDACs results in up-regulation of IFI16 expression and provide evidence for the idea that the expression of IFI16 gene is silenced by mechanisms involving acetylation of proteins.

Our observations provide a molecular basis for defects in the expression of IFI16 gene in prostate cancer cell lines and predict that reduced expression of IFI16 protein in prostate cancer cells contributes to increased expression of AR protein. Because increased expression of AR in prostate cancer cells is thought to contribute to increased cell proliferation and survival (26, 30), our observations will help in understanding the regulation and role of AR in the development and progression of human prostate cancer.

Materials and Methods

Cell Lines, Culture Conditions, and Treatments

LNCaP and DU-145 human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (high glucose) culture medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum and antibiotics. Subconfluent cultures of cells were treated with 5-aza-dC (5 μmol/L) or the indicated concentrations of HDAC inhibitor TSA (in ethanol) alone or CGK1026 (in DMSO) alone, or together with human IFN-α (1,000 units/mL) and IFN-γ (20 ng/mL) for the duration indicated. LNCaP cells were also treated with anisomycin (20 μmol/L) in DMSO; Calbiochem, San Diego, CA) or JNK inhibitor SP600125 (20 μmol/L in DMSO) for the indicated time.

Plasmids and Nucleofections

Mammalian expression vector (pCMV6-JAK1) to express human JAK1 protein was purchased from OriGene Technologies, Inc. (Rockville, MD). LNCaP cells were nucleofected with pCMV6-JAK1 or an empty (pCMV) plasmid using Nucleofector-II device (Amaxa Biosystems, Nattermannallee-1, Germany) and VCA-1001 nucleofection kit (program T-009) as suggested by supplier. To knock down the expression of IFI16 in LNCaP cells, cells were either nucleofected with control siRNA (Silencer Negative Control #1, Ambion, San Antonio, TX) or siRNA IFI16 as described previously (18).

Immunoblotting and Antibodies

Total cell lysates were prepared using a modified radioimmunoprecipitation assay protein extraction buffer as described previously (5). The lysis buffer was supplemented with complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostic Corporation, Indianapolis, IN). Cell lysates containing approximately equal amounts of protein were subjected to immunoblotting using the optimized conditions to detect low levels of IFI16 protein as described previously (19). In brief, proteins were transferred to Immobilon-P (pore size, 0.45 μm; Millipore, Bedford, MA) transfer membranes using the transfer buffer with 0.01% SDS (w/v). After the transfer, membranes were rinsed with PBS and blocked with 5% nonfat dry milk in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.01% Tween 20) for 1 to 2 h at room temperature. The blots were incubated overnight with anti-IFI16 antibodies (1-2 μg/mL) in TBST at 4°C. Binding of anti-IFI16 to membrane was detected by incubation of blot with secondary anti-mouse antibodies conjugated to horseradish peroxidase (Amersham, Piscataway, NJ). The blots were developed using enhanced chemiluminescence developing system (Amersham) and enhanced chemiluminescence Hyperfilm. Mouse monoclonal antibodies (clone 1G7), to detect all three forms of IFI16 protein, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to detect AR, glyceraldehyde-3-phosphate dehydrogenase, JAK1, STAT1, c-Jun, phosphorylated c-Jun, and PARP were purchased from Santa Cruz Biotechnology. Antibodies to detect OAS1 (Abgent, San Diego, CA), phosphorylated STAT1 (Tyr701, Cell Signaling Technology, Danvers, MA), and HSP90 (Stressgen, Victoria, Canada) were purchased from the indicated sources.

RT-PCR

Total RNA was isolated from control untreated or TSA-treated LNCaP cells using Trizol (Invitrogen Life Technologies) method. RNA preparations containing equal amounts of RNA were used to synthesize cDNA using SuperScript First-Strand cDNA synthesis system (Invitrogen), as suggested by the supplier. The cDNA preparations containing equal amount of cDNA were used to do PCR using IFI16-specific primers (5′-primer, 5′-ccagagaagctaactg-3′; 3′-primer, 5′-tagagaagctgcttgagttcatactg-3′) that allowed amplification (total of 40 cycles) of cDNAs for all three (A, B, and C) isoforms of the IFI16 gene splice variants. The PCR primers amplified the cDNA fragment of 685 bp that corresponded to nucleotides 1768 to 2453 in the IFI16 cDNA. The PCR primers for the amplification of the human AR gene have been described previously (17). A SuperArray (Frederick, MD) MultiGene-12 RT-PCR profiling kit (PH-009B) for IFN-activatable genes was used as suggested by the supplier to determine alterations in the expression of IFN-activatable genes after TSA treatment of LNCaP cells. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis.

Gel Mobility Shift Assays

LNCaP cells were either treated with TSA or, as a control, left untreated for the indicated time. The total cell extracts were prepared for gel mobility shift assays as described previously (37). Oligonucleotide containing the wild-type consensus sequence to bind the AP-1 transcription factor was purchased from Santa Cruz Biotechnology. The oligonucleotide containing the AP-1 DNA-binding consensus sequence (5′-TCAGCTAATCAATTGACTCAACCA-3′) and the complementary sequence in the regulatory region of the IFI16 gene (see ref. 25) was synthesized. The oligonucleotides were radiolabeled with polynucleotide kinase and purified as described previously (37). The gel mobility shift assays for AP-1 transcription factor were done as described previously (37).
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Report Assays

Luciferase reporter assays were done as described previously (5). In brief, subconfluent cultures of LNCaP cells were nuclease-treated with AP-1–luc or IFI16-luc reporter plasmid (1.8 µg) along with 0.2 µg pRL-TK plasmid using Nucleofector-II device. LNCaP cells were nuclease-treated as suggested by the supplier using nuclease kit VCA-1001 (program T-009). If so indicated, cells were treated with TSA at the indicated concentration for 24 h. Forty hours after nuclease-treatments, the firefly luciferase and Renilla luciferase activities were determined (in triplicates) using Dual-Luciferase Reporter Assay kit (Promega, Madison, WI). The firefly luciferase activity was normalized to Renilla luciferase to control for variations in nuclease-treatments. The luciferase activity in control vector-transfected cells was considered as 1. SE was calculated using three experimental readings.

Flow Cytometry

Adherent as well as floating cells were pooled and stained with propidium iodide to analyze for accumulation of sub-G₀ cells by flow cytometry as described previously (5).

References

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