Direct Transcriptional Activation of Promyelocytic Leukemia Protein by IFN Regulatory Factor 3 Induces the p53-Dependent Growth Inhibition of Cancer Cells

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Abstract

IFN regulatory factor 3 (IRF3) is a transcriptional factor that plays a crucial role in activation of innate immunity and inflammation in response to viral infection, and is also involved in p53-dependent inhibition of cell growth. Although functional activation of IRF3 by viral infection is relatively well documented, the biological role and regulatory mechanism underlying cell growth inhibition by IRF3 are poorly understood. Here, we show a novel regulatory pathway connecting IRF3-promyelocytic leukemia protein (PML)-p53 in primary and cancer cell lines. Overexpression of IRF3 induces p53-dependent cell growth inhibition in cancer cell lines with normal p53 activity. In addition, doxycycline-induced expression of IRF3 in U87MG cells inhibits tumor growth in nude mice in vivo. IRF3 is found to increase expression of PML by a direct transcriptional activation as determined by PML-promoter-luciferase and chromatin immunoprecipitation assays. When PML is depleted by RNA interference-mediated knockdown, IRF3 fails to increase p53 acetylation and its transcriptional activity. Taken together, the results of the present study indicate that direct transcriptional activation of PML by IRF3 results in the p53-dependent growth inhibition of normal and cancer cells in vitro and in vivo, which is suggestive of a novel regulatory network between the innate immune response and tumor suppression. [Cancer Res 2007; 67(23):11133–40]

Introduction

The innate immune response, the first line of defense against a variety of pathogens, is mainly regulated by the synthesis and secretion of type I IFNs, such as IFN-α and IFN-β, which play crucial roles in antiviral, antibacterial, and antiproliferative functions (1). Recent studies have uncovered two signaling pathways that activate the host innate immunity against viral infection. One of the pathways uses members of the Toll-like receptor family to detect viruses that enter the endosome by endocytosis. The other antiviral pathway uses a RNA helicase, RIG-I, as the receptor for intracellular viral double-stranded RNA. Both the Toll-like receptor- and RIG-1-mediated signaling pathways ultimately lead to the activation of the transcription factors, nuclear factor-κB (NF-κB) and IFN regulatory factor 3 (IRF3), to induce IFN production (2).

The promyelocytic leukemia (PML) protein was originally identified as part of the oncogenic fusion protein PML-retinoic acid receptor α produced by the chromosomal translocation t(15;17) associated with acute promyelocytic leukemia (3). IFN is the best characterized inducer of PML mRNA expression through Janus-activated kinase/signal transducers and activators of transcription signaling, IFN-stimulated response elements (ISRE), and IFN-γ-activated sites (GAS) in the PML promoter (4). PML nuclear bodies (PML-NB) in the nucleus seem to have multiple functions as a growth and tumor suppressor (5), a mediator of multiple apoptotic signals (6, 7), and a coactivator and corepressor of various transcription factors (8–10). Currently, p53, pRB, Daxx, BLM, CBP, Spectrin, SUMO-1, Sp100, and Sp140 have been identified as components of the PML-NBs (8, 11–16).

The p53 tumor suppressor is activated in response to many stimuli, including hyperproliferative signals, DNA-damaging agents, and viral infection (17), and functions as a transcription factor to promote several antiproliferative responses via cell cycle checkpoints, cellular senescence, and apoptosis (18). The rapid induction of p53 is achieved through posttranslational modifications leading to its stabilization and activation. In particular, acetylation of lysine residues in the COOH terminus of p53 by acetyltransferases such as p300/CREB–associated factor and CBP/p300 (19–21) increases its DNA-binding and transcriptional activities (22–24). Moreover, acetylation has been linked to the stability of p53 and to the recruitment of coactivators (21, 25).

We previously showed that IRF3 inhibits cell proliferation and induces cellular senescence by activating p53 function in normal cells (26). However, the biological function and regulatory mechanism of IRF3 in cancer cell growth have not been well documented yet. Here, we found that IRF3 induces expression of PML, which subsequently increases the acetylation and transcriptional activity of p53. In the present study, we have established that PML acts downstream of IRF3 and upstream of p53 to inhibit the growth of cancer cells in vitro and in vivo.
Materials and Methods

Cells and culture conditions. Human BJ fibroblast cells and human cancer cell lines (U87MG, U-2OS, HepG2, MCF7, HT1080, and Hep3B) were purchased from American Type Culture Collection. BJ cells and cancer cell lines as well as astrocytes and mouse embryonic fibroblasts (MEF) derived from p53−/− mice were maintained in DMEM/high glucose medium enriched with 10% fetal bovine serum (Hyclone), 1% penicillin and streptomycin (Life Technologies), and 2 mmol/L γ-glutamine (Life Technologies). To determine cell growth rates, cells were plated at a density of 2.5 × 10⁴ to 5.0 × 10⁴ per six-well plate. Three or five days after cell plating, cell numbers were counted using a hemocytometer. For a low-density seeding assay, 5 × 10³ cells were seeded into a 10-cm dish, cultured in 10% fetal bovine serum–DMEM for 14 days, and stained with Giemsa stain–modified solution (Fluka). To stimulate IRF3-dependent innate immune signaling, cells were treated with lipopolysaccharide (LPS; 1 μg/mL, Sigma) and the copolymer of polyinosinic and polycytidylic acids (poly IC; 10 μg/mL, Amersham).

Plasmids and retroviral infection. Using reverse transcription-PCR (RT-PCR) amplification with a human IRF3-specific primer set, the full-length human IRF3 cDNA fragment was cloned into pcDNA3.1-Neo vector (Invitrogen), pRevTRE-Hygro retroviral vector (Clontech), and pBABE-Puro retroviral vector. IRF3-small interfering RNA (siRNA) wild-type form, IRF3-siRNA mutant form (one nucleotide of IRF3-siRNA wild-type form was

Figure 1. IRF3 inhibits cancer cell proliferation and tumorigenesis.

A, low-density seeding assay of U87MG (p53 positive), U-2OS (p53 positive), and Hep3B (p53 negative) infected with pBABE-Puro and pBABE-IRF3-Puro. B, expression of IRF3 and α-tubulin proteins (loading control) in pRevTRE-IRF3–infected U87MG and U-2OS cells grown in the absence or presence of doxycycline (DOX; 1 μg/mL) for 48 h.

C, representative photographs (>40 magnification) and relative foci numbers of the pRevTRE-IRF3–infected U87MG and U-2OS cells grown in soft agar with or without doxycycline (1 μg/mL) for 3 wk. Columns, mean of triplicate experiments; bars, SD. *P < 0.05, statistical significance between paired samples. D, representative photographs and tumor growth rates of nude mice that were s.c. injected with pRevTRE-IRF3–infected U87MG cells and were treated with (bottom) or without (top) doxycycline (1 mg/mL).
mismatched), IFN-β-siRNA, and PML-siRNA were cloned into pSUPER-PURO and Hygro [the constitutive RNA interference (RNAi) expression vectors] according to the instructions of the manufacturer (Oligoengine). The target sequences were IFR3-siRNA wild-type form: AAGACATTCTG-GATGAGTTAC; IFR3-siRNA mutant form: AAGTACATTCTGAGATGTTAC; IFN-β-siRNA: AAGCTCTTGTGCCATGGTAAT; PML-siRNA: AGACCAAA-CACACTCTGTCG. BJ, U87MG, U-2OS, HT1080, p53+/−, astrocyte, p53/C0 MEF, or p53−/− MEF cells were infected with retrovirus produced from the PT67 amphotropic packaging cell line (Clontech) transfected with retroviral vectors (pBABE-IRF3-Puro, pRevTRE-IRF3-Hygro, pRevSuper-IRF3-siRNA wild-type form-Puro, pRevSuper-IRF3-siRNA-mismatch form-Puro, and pRevSuper-IFN-β-siRNA-Puro, and pRevSuper-PML-siRNA-Puro). The cells plated 24 h earlier at 10^5 cells/10-cm dish were transfected by reseding them with prefiltered (0.45 um) retroviral supernatant containing 6 mg/ml of polybrene (Sigma). This step was repeated twice without polybrene. Twelve hours after final infection, cells were subjected to drug selection for 5 to 14 days.

Reverse transcription-PCR. Total RNA was isolated from cells using TRIzol (Life Technologies) according to the instructions of the manufacturer. For semiquantitative RT-PCR, 3 mg of DNase I–treated RNA were converted to cDNA with Superscript II reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. A portion (1 μl) of the reverse transcriptase reaction was then used to amplify IFN-α, IFN-β, IP-10, PML, IRF3a, and p53 and NF-κB transcriptional activity as well as PML promoter in U87MG cells infected with pBABE-Puro. pBABE-IRF3-Puro content was determined by transfection of pGL3-p53CBS-mut plasmid (p53 consensus-binding sequences—mutant form as negative control), pGL3-p3CBS plasmid (p53 consensus-binding sequences—normal form), pGL3-p21 plasmid (p53-binding sequences from p21WAF1 promoter), and pGL3-Mdm2 plasmid (p3-binding sequences from Mdm2 promoter).)

Western blot and immunoprecipitation. Whole-cell extracts were prepared using radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 7.4)] containing protease and phosphatase inhibitors (Roche). Cytosolic, nuclear, and membrane proteins were prepared using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) and following the protocol of the manufacturer. Cell extracts were quantitated using the Bradford assay procedure according to the instructions of the manufacturer. Protein in the extracts (30–100 μg) was separated by 4% to 12% gradient or 10% SDS-PAGE NuPAGE gel (Invitrogen) and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% nonfat milk and incubated with anti-IRF3 (FL-425, Santa Cruz Biotechnology), anti-p53 (DO-1, Santa Cruz Biotechnology for normal p53), Ab-1, onconege for p53DD protein; Ab-1, onconege for p53DD protein, anti-p21WAF1 (C-19, Santa Cruz Biotechnology), anti-Bax (N-20, Santa Cruz Biotechnology), anti-PML (P-1, M3, Santa Cruz Biotechnology for human PML protein), 05-718, Upstate for mouse PML protein), anti-histone H3 (06-755, Upstate), anti-APC (C-20, Santa Cruz Biotechnology), anti-β-actin and anti-histone H3 (06-755, Upstate), anti-APC (C-20, Santa Cruz Biotechnology), anti-β-actin (Pierce). Immunoprecipitation assays were conducted using whole-cell lysates (200 μg), anti-p53 antibody, and Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Western blot analysis was carried out using horseradish peroxidase-conjugated anti-secondary IgG (Pierce) antibody and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Immunoprecipitation assays were conducted using whole-cell lysates (200 μg), anti-p53 antibody, and Protein A/G PLUS-Agarose (Santa Cruz Biotechnology), and the acetylated and phosphorylated p53 proteins were detected with anti-acetyl-p53 antibody (∼48kDa, Upstate), and anti-phosphorylated p53 antibody (Ser15, 16G8, Cell Signaling) as described above.

Luciferase-reporter activity assay. The relative luciferase activities of p53 and NF-κB transcriptional activity as well as PML promoter in U87MG and p53−/− astrocyte cells infected with pBABE-Puro and pBABE-IRF3-Puro were determined by transfection of pGL3-p53CBS-mut plasmid (p53 consensus-binding sequences-mutant form, as negative control); pGL3-p53CBS plasmid (p53 consensus-binding sequences-normal form); pGL3-p21 plasmid (p53 binding sequences from p21WAF1 promoter); pGL3-Mdm2 plasmid (p3-binding sequences from Mdm2 promoter). The luciferase reporter activity was measured by Viability Assay (Promega). Transfection efficiency was normalized with the activity of Renilla luciferase, which was cotransfected with the aforementioned pGL3-luciferase-reporter constructs.

Indirect immunofluorescence. Cells grown in eight-chamber slides (BD Biosciences) were fixed with precooled methanol for 10 min at −20°C. After...
two washes with PBS, cells were blocked with PBS supplemented with bovine serum albumin (Sigma) and 0.1% NP40 (Fluka) for 20 min to reduce nonspecific binding. Cells were then incubated with primary anti-PML antibody (Santa Cruz Biotechnology), anti–NF-κB p65 (c-20, Santa Cruz Biotechnology), anti–acetyl-p53 antibody (Upstate), and anti–phosphorylated p53 antibody (Cell Signaling) for 1 h at 20°C. After two washes with PBS, cells were incubated with a fluorescein-conjugated secondary antibody (Pierce). Nuclei were then stained with 4',6-diamidino-2-phenylindole (1 μg/mL) for 10 min, and cells were mounted using Vectashield mounting medium (Vector). Fluorescence images were obtained by confocal microscopy (Zeiss).

**Cell death analysis.** The trypan blue exclusion method was used to assess the viability and death rates of cells grown for 24 h in the absence or presence of the various DNA-damaging agents [30 μg/mL 1,3-bis(2-chloroethyl)-1-nitrosourea, Sigma; 500 nmol/L staurosporine, Calbiochem; 100 μmol/L doxorubicin, Sigma].

**Chromatin immunoprecipitation assays.** The chromatin immunoprecipitation (ChIP) assay was done according to the protocol of the manufacturer (Upstate) with a minor modification. After one round of immunoprecipitation with anti-IRF3 (Santa Cruz Biotechnology) and anti-mouse IgG (Pierce), the precipitates were precipitated, and dissolved in TE buffer. The purified DNA was analyzed by PCR (36 cycles; 30 s at 95°C, 30 s at 58°C, 30 s at 72°C) with primers that detect specific sequences of upstream genomic regions of PML gene containing PML promoter 2 (nucleotide number −621 to −4; primers, F: CAGTGTGAACGGATGAATGG, R: CATGGACCCCAGCTTAGTTT).

**Soft agar assay.** To assess anchorage-independent growth, the U87MG and U-2OS cells (1×10⁴) infected with pRevTRE-IRF3-Hygro were cultured into six-well soft agar dishes (1.6% and 0.7% bottom and top agar, D). They were cultured for 3 weeks, and cells growing on the surface were counted.

**Results**

**IRF3 inhibits cancer cell proliferation and tumorigenesis.** Because IRF3 is an inducer of cell growth inhibition and cellular senescence through the activation of p53 tumor suppressor in normal somatic cells (26), we investigated the biological function of IRF3 in human cancer cell lines. Similar to the normal human fibroblast BJ cells, overexpression of IRF3 in U87MG and U-2OS cells (both p53 positive) was shown to decrease cell growth by activating a p53 tumor suppressor, whereas IRF3 overexpression failed to inhibit growth of Hep3B cells (p53 negative; Fig. 1A (Supplementary Fig. S1A)). Moreover, in contrast to p53⁻/⁻ MEFs, cell proliferation of p53 wild-type MEF was markedly decreased by ectopic expression of IRF3 (Supplementary Fig. S1B). These results suggest that IRF3-induced cell growth inhibition is dependent on p53 activity, and is conserved in human and mouse cells. The soft agar assay using the doxycycline-inducible expression system of IRF3 (Fig. 1B) showed that dramatic inhibition of growth of both U87MG and U-2OS cancer cells upon IRF3 expression is anchorage

**S.c. tumorigenicity assays.** For transplantation experiments, 1×10⁶ cells were transplanted s.c. into BALB/c nu/nu nude mice (n = 5). To induce IRF3 expression, mice received doxycycline (1 mg/mL) in their drinking water. Injected mice were observed every 3 days. S.c. tumors were grossly visible at the site of injection after 3 to 4 weeks.

**Statistics.** Data were analyzed statistically using a two-tailed Student’s t test. The level of statistical significance stated in the text was based on the P values. P < 0.05 was considered statistically significant.

**Figure 3.** Induction of PML expression by IRF3. A, expression of IP-10, PML, and GAPDH (loading control) mRNAs in the pRevTRE-IRF3⁻infected U87MG cells grown in the absence or presence of doxycycline (1 μg/mL) for 48 h or in the absence of doxycycline for 48 h after 48 h doxycycline. B, expression of IRF3, PML, and α-tubulin proteins (top) as well as PML and GAPDH mRNA (bottom) in BJ fibroblast cells infected with pBABE-Puro and pBABE-IRF3-Puro. C, expression of IP-10, PML, and GAPDH mRNAs in the p53DD-overexpressing U87MG cells infected with pBABE-Puro and pBABE-IRF3-Puro (top). Expression of PML and α-tubulin proteins in p53⁻/⁻ astrocytes (middle) and p53⁻/⁻ MEFs (bottom) infected with pBABE-Puro and pBABE-IRF3-Puro. D, representative immunofluorescence images (×400 magnification) showing PML-NBs in the U87MG, BJ fibroblast, p53⁻/⁺ MEF, and p53⁻/⁻ MEF cells infected with pBABE-Puro and pBABE-IRF3-Puro.
Inhibition of cell proliferation by IRF3 is dependent on p53 activity in cancer cell lines. To confirm that IRF3-induced inhibition of cell growth occurs through the activation of p53 tumor suppressor, we examined the expression levels of target genes regulated by p53 in U87MG cells. As shown in Fig. 2A, the induction of IRF3 expression in U87MG cells for 2 days with doxycycline increased the expression of p53-downstream target genes such as p21\(^{WAF1}\) and Bax, whereas the reduction of IRF3 levels by doxycycline withdrawal down-regulated p21\(^{WAF1}\) and Bax expression. Furthermore, the luciferase reporter assay with promoters containing p53-binding sites in IRF3-overexpressing U87MG cells showed that the transcriptional activity of p53 increased in an IRF3-dependent manner (Fig. 2B), suggesting that activation of the p53 signaling pathway should be directly regulated by IRF3. To further determine biological significance of the IRF3-dependent activation of p53, we have transduced IRF3 into U87MG and U-2OS cells expressing a dominant negative p53 (p53DD) that is known to inhibit the activity of wild-type p53 (Fig. 2C; ref. 27). As shown in Fig. 2C, overexpression of p53DD in the U87MG and U-2OS cells dramatically suppressed the growth-inhibitory activity of IRF3, which was observed in IRF3-overexpressing parental U87MG and U-2OS cells (Supplementary Fig. S1A). As expected, expression of p21\(^{WAF1}\) in the p53DD-overexpressing U87MG and U-2OS cancer cells was not increased by IRF3 (Fig. 2C).

Because the activation of p53 is mainly regulated by posttranslational modifications such as phosphorylation and acetylation leading to its stabilization and activation, we examined whether p53 is hyperphosphorylated or hyperacetylated in the cells overexpressing IRF3. The level of acetylated p53 protein was dramatically increased in U87MG cells upon induction of IRF3 expression with doxycycline treatment, as determined by Western blotting and immunofluorescence (Fig. 2D), whereas the level of phosphorylated p53 protein was slightly increased in these cells (Fig. 2D). Meanwhile, depletion of IRF3 by RNAi-mediated knockdown in the U87MG cells decreased cell death induced by a variety of DNA-damaging agents (Supplementary Fig. S2A, S2B, and S2C). Taken together, these results indicate that IRF3 might exert an antiproliferative effect by activating p53 function and increasing its acetylation in cancer cell lines.

IRF3 is an up-regulator of PML. Previous studies have shown that, upon overexpression of PML, p53 is recruited to the PML-NBs and acetylated at its lysine 382, leading to enhancement of its transcriptional activity and that PML thus acts as an activator of p53 (28). Therefore, we examined expression of PML in U87MG-RevTRE-IRF3 cells to determine the possible mechanisms underlying the IRF3-dependent activation of p53. As shown in Fig. 3A, the levels of PML mRNA and IP-10 mRNA, an IRF3-downstream target gene, were increased by induction of IRF3 expression in the U87MG-RevTRE-IRF3 cells upon treatment with doxycycline when compared with U87MG-RevTRE-IRF3 cells untreated with doxycycline. As expression of IRF3 was reduced independent (Fig. 1C). Interestingly, endogenous IRF3 protein was found to be localized in the cytosol, nucleus, and cell membrane. However, overexpression of IRF3 showed increased localization of the protein in the nucleus (Supplementary Fig. S1B). To validate the tumor-suppressive potential of cells expressing IRF3 in vivo, we injected U87MG-pRevTRE-IRF3 cells s.c. into nude mice in the absence or presence of doxycycline (1 \(\mu\)g/mL). In vivo induction of IRF3 expression in U87MG cells containing pRevTRE-IRF3 significantly decreased tumor formation (Fig. 1D). Taken together, these results indicate that IRF3 should have tumor-suppressive functions in vitro and in vivo.
following doxycycline withdrawal in these cells, the PML mRNA level was shown to decrease, suggesting that IRF3 directly regulates the expression of PML. Moreover, overexpression of IRF3 was also shown to increase the levels of PML mRNA and its protein in normal human fibroblast BJ cells (Fig. 3B).

Because p53 is also known to induce the expression of PML, we assessed whether IRF3 can induce expression of PML in several p53-deficient cells. As shown in Fig. 3C, overexpression of IRF3 obviously increased expression of PML mRNA and its protein in p53DD-overexpressing U87MG cells and in both astrocytes and MEFs derived from p53-knockout mice. As determined by indirect immunofluorescence, PML-NB formation was found to dramatically increase in IRF3-overexpressing U87MG, BJ, p53+/− MEF, and p53−/− MEF cells compared with their counterpart control cells (Fig. 3D). These results indicate that p53 is not a positive regulator for PML expression and might be downstream of PML.

Although IFN-α is the best-characterized PML regulator to induce PML mRNA expression, we excluded IFN-α as an upstream regulator of PML because expression of IFN-α was not detected in U87MG cells following induction of IRF3 or LPS treatment (Fig. 4A). We also established a LN229 glioblastoma cell line (LN229−pSRP-IFN-β-RNAi) in which IFN-β was depleted by RNAi-mediated knockdown (Fig. 4A). Upon induction of IRF3, increased p21WAF1 protein, PML mRNA, and PML-NBs formation were observed in IFN-β-knockdown LN229 cells (Fig. 4B and C). Using HT1080 cells that have genetic defects in the IFN-signaling pathway (29), we further confirmed that overexpression of IRF3 in these cells results in a marked increase of PML-NBs and induces a senescent-like phenotypic change as observed in the BJ fibroblast cells (ref. 26; Fig. 4D). These results indicate that induction of PML expression by IFN signaling and overexpression of IRF3 might be regulated differently. Furthermore, we found that human H1N1 influenza virus (A/PR/8/34) induces a significant increase in the level of PML mRNA in BJ fibroblast cells compared with IRF3-knockdown BJ cells (Supplementary Fig. S3A) and slightly delays the H1N1-induced cell death in the IRF3-knockdown BJ cells (data not shown).

PML is a direct transcriptional target of IRF3. Up-regulation of PML expression by IRF3 suggested that PML might be a direct transcriptional target of IRF3. We thus examined the IRF3-dependent PML promoter activity and the binding affinity of IRF to PML promoter using PML-promoter-luciferase and ChIP assays, respectively. As shown in Fig. 5A, three different regions of human PML promoter were inserted into pGL3-basic vector: PML-P1 (−1,344 to −621), PML-P2 (−626 to −4 containing both GAS and ISRE elements), and PML-P3 (−36 to +1,427). Consequently, an ~2.5-fold increase of luciferase activity in PML-P2 was induced in the IRF3-overexpressing p53−/− astrocytes compared with control vector–infected p53−/− astrocytes, whereas no effect on luciferase activity was observed from PML-P1 and PML-P3 constructs transfected to these cells (Fig. 5B). Using a ChIP assay, we determined that endogenous IRF3 in U87MG cells was bound to the PML-P2 region (Fig. 5C, inset) but not to the PML-P1 and PML-P3 regions (data not shown). In IRF3-overexpressing U87MG cells, binding of IRF3 in the PML-P2 region showed an 8-fold increase compared with that of control vector–infected U87MG cells (Fig. 5C). Because NF-κB is one of the distinct pathways for the innate immune response to viral infection with IRF3 (30), we also investigated expression and subcellular localization of NF-κB and its transcriptional activity by immunofluorescence and promoter-reporter gene assays. However, the results showed that there were no evident differences in NF-κB activity between control vector– and IRF3-overexpressing U87MG cells (Supplementary Fig. S3B and S3C). We used a DNA binding domain–deleted isoform of IRF3 (IRF3a; an alternatively spliced protein deficient in the transcription activity; ref. 31) to address whether cell growth inhibition and PML expression by IRF3 are required for its transcriptional activity. In contrast to IRF3, overexpression of IRF3a failed to inhibit proliferation of the NIH-3T3 cells (Supplementary

Figure 5. PML is a direct transcriptional target of IRF3. A, a schematic diagram depicting the genomic regions of human PML spanning −1,344 bp upstream and +1,427 downstream of the predicted PML transcriptional start site (+1). The GAS and ISRE boxes represent previously identified sequences. Sequences corresponding to −1,344 to −621 (PML promoter 1), −621 to −4 (PML promoter 2), and −36 to +1,427 (PML promoter 3) were used for the luciferase reporter assay. B, relative luciferase activity of three different reporter-gene constructs containing PML promoters 1, 2, and 3 in the p53−/− astrocytes infected with pBabe-Puro and pBabe-IRF3-Puro. Columns, mean of triplicate experiments; bars, SD. *, P < 0.05, statistical significance between paired samples. C, relative PML promoter-binding rate of IRF3 determined by ChIP. Inset, representative results of semiquantitative PCR with immunoprecipitated chromatin by IRF3 antibody and a primer set (arrows in A) that could amplify PML promoter sequences containing GAS and ISRE (−340 to +3). The input lane corresponds to 1% of total chromatin used for the ChIP reactions. The ChIP with mouse IgG was used as negative control. Columns, mean of triplicate experiments; bars, SD. *, P < 0.05, statistical significance between paired samples.
Fig. S4A) and relatively enhanced growth of the NIH-3T3 cells (Supplementary Fig. S4B). Furthermore, IRF3a did not induce expression of p21WAF1 and PML in the U87MG cells (Supplementary Fig. S4C). Taken together, these results indicate that PML is a direct target for IRF3 and that the PML-P2 region of the promoter is largely responsible for the ability of IRF3 to up-regulate the expression of PML.

**Activation of p53 by IRF3 is dependent on PML.** Because PML is known to up-regulate p53 activity by activating p53 acetylation, we determined whether activation of p53 by IRF3 is regulated by overexpression of PML by IRF3. Using an RNAi-mediated PML knockdown system, PML was shown to be significantly downregulated in the presence or absence of poly IC, an inducer of PML (Fig. 6A). Overexpression of IRF3 did not induce formation of the PML-NBs (Fig. 6B) and did not induce expression of p21WAF1 (Fig. 6C) in the PML-depleted U87MG cells compared with their counterpart cells. Additionally, IRF3-induced increase in p53 acetylation was relatively suppressed by knockdown of PML (Fig. 6C and D). Taken together, these results delineate that functional activation of p53 tumor suppressor by IRF3 should be regulated by PML-dependent acetylation of p53 protein.

**Discussion**

**IRF3 as a potential tumor suppressor.** Although IRF3 is part of a family of IFN regulatory transcription factors that contribute to cellular antiviral responses, mounting data suggest that IRF3 and its signaling pathway might have a potential for antioncogenic function. The expression of a dominant negative IRF3 mutant (IRF3a) that specifically inhibits expression of IRF3 target genes facilitated oncogenic transformation in vitro and in vivo (32). Prolyl isomerase Pin1, a potential oncogene (33), was shown to negatively regulate the IRF3-dependent innate antiviral response by stimulating proteasome-dependent degradation of IRF3 (34). IRF3 inhibits cell proliferation and induces cellular senescence by activating p53 function in normal cells (26). In addition, we show here that IRF3 should be a novel inducer of the p53-dependent inhibition of cell growth through direct transcriptional activation of PML in cancer cells, which is suggestive of a IRF3 cell autonomous tumor-suppressive function.

The IRF3 complex translocates to the nucleus, where it activates promoters containing IRF3-binding sites found in the IFN-β, CCL5/RANTES, and CXCL10/IP-10 genes. Although IFN-β, CXCL10/IP-10, and CCL5/RANTES have all been reported to act as primary response genes after activation of IRF3 in LPS-stimulated murine B cells (35), IFN-β was not activated by IRF3 in U87MG and U-2OS cancer cells, whereas CXCL10/IP-10 was activated; these findings suggest that expression of cytokines by IRF3 is differentially regulated in the different cell types. It has been shown that IRF3 exhibits a tumor-suppressive function by means of cell nonautonomy, such as through the induction of cytostatic cytokines. For instance, IRF3-dependent inhibition of tumor growth was found to be correlated with elevated expression of chemokines such as MIP-1, CCL5/RANTES, and CXCL10/IP-10 in vivo and dramatic induction of cytokines such as IFN-β, tumor necrosis factor-α, and...
interleukin 6 in vitro in B16 melanoma tumors (36). Previous data revealed not only a novel mechanism by which CXCL10/IP-10 inhibits viral replication through the induction of host cell death via a p53-mediated apoptotic pathway but also showed a mechanism by which IP-10 inhibits tumor cell growth in HeLa cells (37). Taken together, these data suggest that IRF3 could directly or indirectly inhibit cell growth, through activation of the PML-p53 pathway or through induction of several cytostatic cytokines and chemokines, respectively.

Transcriptional regulation of PML by IRF3. It is well documented that PML promoter possesses both the ISRE and NF-κB sites. Although all IFNs (α, β, and γ) sharply induce PML expression leading to a marked swelling of the PML-NBs (4, 38), IFN-β, the best characterized target gene induced by IRF3, is the most powerful inducer of PML expression and PML-NB formation. However, we could exclude a potential role of IFN-γ in the direct activation of PML expression by IRF3, because U87MG and U-2OS cancer cells (IFN-γ expression is not activated by IRF3) as well as LN229 cells (IFN-β expression is down-regulated by an IFN-β-specific RNAi system) were shown to express induction of PML and formation of PML-NBs by IRF3. Furthermore, using ChIP and luciferase-reporter assays, we showed that PML was a direct transcriptional target of IRF3 and that both ISRE and GAS elements of PML, but not ISRE alone, seem to be required for transcriptional activation of PML by IRF3. These findings indicate that IRF3 might need other transcription factors to regulate the expression of PML. For example, expression of IFN-β is regulated by enhancerosome, a multiprotein complex composed of activating transcription factor 2/cJun, NF-κB, and IRF3 that is assembled at the IFN-β enhancer in response to a viral challenge (39). Although NF-κB and IRF3, the two major factors for triggering the innate immune response, cooperate to control the expressions of a variety of cytokines and chemokines due to viral infection, and NF-κB alone has been shown to modulate the p53-mediated apoptosis pathway (40), the activation of the NF-κB might not be necessary for PML-dependent activation of p53 by IRF3. However, it is plausible that activation of both NF-κB and IRF3 should accelerate PML-dependent p53 activation and tumor-suppressive function. In conclusion, our results indicate that direct transcriptional activation of PML by IRF3 results in the p53-dependent growth inhibition of cancer cells, which is suggestive of a novel regulatory network between the innate immune response and tumor suppression.

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Direct Transcriptional Activation of Promyelocytic Leukemia Protein by IFN Regulatory Factor 3 Induces the p53-Dependent Growth Inhibition of Cancer Cells

Tae-Kyung Kim, Joong-Seob Lee, Se-Yeong Oh, et al.


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