Promyelocytic Leukemia Protein Sensitizes Tumor Necrosis Factor α-Induced Apoptosis by Inhibiting the NF-κB Survival Pathway*

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The promyelocytic leukemia protein (PML) is a growth/tumor suppressor essential for induction of apoptosis by diverse apoptotic stimuli. The mechanism by which PML regulates cell death remains unclear. In this study we found that ectopic expression of PML potentiates cell death by apoptosis in the tumor necrosis factor α (TNFα)-resistant cell line U2OS and other cell lines. Treatment with TNFα significantly sensitized these cells to apoptosis in a p53-independent manner. PML/TNFα-induced cell death is associated with DNA fragmentation, activation of caspase-3, -7, and -8, and degradation of DNA fragmentation factor/inhibitor of CAD. PML/TNFα-induced cell death could be blocked by the caspase-8 inhibitors CrmA and c-FLIP but not by Bcl-2. These findings indicate that this cell death event is initiated through the death receptor-dependent apoptosis pathway. PML is a transcriptional repressor of NF-κB by interacting with RelA/p65 and prevents its binding to the cognate enhancer through the C terminus. Comunmunoprecipitation and double-color immunofluorescence staining demonstrated that PML physically interacts with RelA/p65 in vitro and the two proteins colocalized at the endogenous levels. Overexpression of NF-κB rescued cell death induced by PML/TNFα. Furthermore, PML−/− mouse embryo fibroblasts are more resistant to TNFα-induced apoptosis. Together these results define a novel mechanism by which PML induces apoptosis through repression of the NF-κB survival pathway.

The promyelocytic leukemia gene (PML)1 was initially identified through its fusion to retinoic acid receptor α (RARα) involved at the breakpoint of t(15,17) (q22;q12) chromosomal translocation in acute promyelocytic leukemia (APL) (1). The PML-RARα fusion protein created by this translocation interferes with the normal function of PML and the RAR/retinoid X receptor pathway and plays an important role in the pathogenesis of APL (2, 3). PML is a nuclear protein localized in discrete subnuclear compartments designated PML nuclear bodies (NBs) or PML oncogenic domains (4). PML is a primary target gene of interferons (IFNs) and is widely expressed in almost all cell lines tested (5). PML is a tumor/growth suppressor that regulates cell cycle progression and induces cell death (6–9). The proapoptotic and growth-suppressing functions of PML were demonstrated in vivo using cells obtained from PML knockout mice. This study showed that PML is required for Fas- and caspase-dependent DNA damage-induced apoptosis in splenocytes and is essential for the induction of programmed cell death (PCD) by Fas, tumor necrosis factor α (TNFα), ceramide, IFNα, IFNβ, and IFNγ (10). PML also induces a caspase-independent cell death when force-expressed in rat embryo fibroblasts (11). How PML exerts its proapoptotic effects remains unknown.

The small ubiquitin-like protein SUMO-1 modifies PML at three lysine residues (12). This modification appears to be essential for the integrity and function of the PML NBs. Several reports (13) documented that modification of PML by SUMO-1 is required for the formation of PML NBs. A PML mutant lacking the SUMO-1 sites did not form PML NBs. Reintroduction of the wild-type PML but not the SUMO-1 mutant PML into the PML−/− mouse embryo fibroblasts (MEFs) led to the reorganization of the PML NBs (14, 15). This study convincingly demonstrated that SUMO-1 modification of PML is necessary for the NB formation. Recent studies (16, 17) also showed that SUMO-1 modification of PML is essential for interaction and regulation of transcriptional repression function of Daxx.

Apoptosis is a genetically controlled suicide process consisting of two phases. The first phase is characterized by a commitment to cell death; the second phase, an execution phase, is characterized by membrane inversion, exposures of phosphatidylserine residues, blebbing, chromatin condensation, and DNA fragmentation. Two major apoptotic pathways, the mitochondria-dependent pathway and the death receptor-mediated pathway, have been well documented (18–20). The activation of an apoptotic pathway does not necessarily result in cell death because nuclear factor NF-κB, which up-regulates antiapoptotic genes that block cell death, is also frequently activated. For example, the activation of TNF receptor results in caspase-8 processing, leading to the induction of cell death, whereas NF-κB activation induced by TNF inhibits cell death (21, 22). Therefore, the sensitivity of cells to apoptotic signals depends on both the NF-κB-mediated...
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survival pathway and the proapoptotic pathways. Consistent with this notion, c-Mye, E1a, and E2F1, which inhibit the NF-κB-mediated signaling pathway, enhanced PCD (23–25).

Our results show that PML induces PCD by a death receptor-mediated apoptotic pathway and sensitizes cells to apoptosis upon treatment with TNFα by inhibiting the NF-κB survival pathway. PML represses NF-κB function by interfering with its binding to the NF-κB target. A loss of PML function in PML−/−MEFs renders cells resistant to TNFα-induced apoptosis. These findings shed new light on the mechanism of PML function as a tumor suppressor and define a novel mechanism by which PML induces apoptosis through repression of the NF-κB survival pathway.

EXPERIMENTAL PROCEDURES

Plasmids Construction—The full-length PMLIV cDNA was obtained from Professor Pierre Chambon (Institut de Chimie Biologique, Strasbourg Codex, France). The inducible expression plasmids pMEP4/PML and pMEP/PML(1–555) were constructed by subcloning the PML cDNA or PML(1–555) into the NotI/XhoI sites of the pMEP4 vector (Invitrogen). The plasmid pMEP4/PML(97−633) was generated by cloning the PML(97−633) DNA fragment from pCDNA3.1/PML(97−633) into the HindIII/XhoI sites. The plasmids pMEP4/HA-p53, pMEP4/HA-CrmA, and pMEP4/c-FLIP were constructed by subcloning the full-length cDNAs amplified by PCR into the pMEP4 vector. The expression plasmids pCDNA3/p65 and pMEP4/p65 were created by subcloning the full-length cDNA of p65/RelA into the BamHI/XhoI sites of pCDNA3 and the HindIII/XhoI sites of pMEP4. pCMVHA-NLS-p53 and pCMVHA-NLS-LacZ were generated by cloning RelA/p53 cDNA and LacZ cDNA, respectively, into the BamHI/XhoI sites of pCMV23T. The pCDNA3.1/PML(97−633) was created by cloning the AvrII/EcoRI DNA fragment into the BamHI and EcoRI sites of pCDNA3.1HisA. The NF-κB-Luc reporter was obtained from Clontech Laboratories, Inc. (Palo Alto, CA).

Cell Cultures and Reagents—The U2OS, 293T, SiHa, Saos2, PML−/−MEF, and PML+/+MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. DFF antibody (66-069), HA monoclonal antibody (mAb), and caspase-8 (05-477) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Caspase-7 (66871A) and caspase-8 antibodies (66231A) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfection and Luciferase Reporter Assay—Cells were cultured to 90% confluency and transfected with the expression plasmids using FuGENE 6 transfection reagent (Roche Diagnostics). For transfection into MEFs, the Effectene reagent (Qiagen, Valencia, CA) was used. Luciferase activity was determined using the luciferase reporter assay according to the manufacturer’s protocol (Promega Corp., Madison, WI).

Generation of Stable Cell Lines—U2OS cells were transfected with each of the plasmids: pMEP4 (negative control), pMEP4/PML, pMEP4/HA-CrmA, pMEP4/HA-p53, pMEP4/c-FLIP, pMEP4/p65, and pMEP4/Bcl-2 with FuGENE 6 (Roche Diagnostics) and then selected with hygromycin (200 μg/ml) for 10 days to establish the stable clones pMEP4/U2OS, pCDNA3/U2OS, pMEP4/CrmA/U2OS, pMEP4/p65/U2OS, and pMEP4/Bcl-2/U2OS, respectively. Inducible expression levels of the respective proteins in each stable cell line were determined by induction with CdSO4 (5 μM) for 20 h followed by immunofluorescent staining and Western blot analysis.

Cell Death Analysis—Cell death was examined by the cell death enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (Roche Diagnostics) or by trypan blue exclusion assay. The TUNEL assay was also performed to determine cell death according to the manufacturer’s protocol (Promega Corp.).

 Colony Forming Assay—A stable cell line in U2OS (105) or the control line (pMEP4 in U2OS) was cultured in 6-well plates in DMEM containing 10% FCS and hygromycin. After 8 h, 5 μM CdSO4 or phosphate-buffered saline (PBS) was added, and the culture was continued for 10 days.

Subcellular Fractionation of Cytoplasmic and Nuclear Proteins—Cytoplasmic protein was prepared by the digitonin extraction method. Cultured cells were washed twice with cold PBS and resuspended in ice-cold digitonin extraction buffer (10 mM Tris, 0.4 M glycine, 2 mM EDTA, 0.1% Triton X-100, 300 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and centrifuged. Cytoplasmic protein was resuspended in 1 ice-cold digitonin extraction buffer (10 mM Tris, 0.4 M glycine, 2 mM EDTA, 0.1% Triton X-100, 300 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. After centrifugation for 10 min at 5,000 × g, the nuclei were resuspended in nuclear extraction buffer (50 mM PIPES, pH 7.4, 0.5% (ν/ν) Triton X-100, 300 mM sucrose, 5 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. After centrifugation for 10 min at 5,000 × g, the nuclei were resuspended in nuclear extraction buffer (50 mM PIPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1% (ν/ν) Triton X-100, 0.5% (ν/v) Nonidet P-40, 10% (ν/v) glycerol). The nuclear fraction was incubated for 30 min on ice and then centrifuged for 5 min (7,800 × g) at 4 °C. The supernatant contained the nuclear proteins.

Immunofluorescence Staining and Confocal Microscopy—Immunofluorescence staining was performed as described in our previous report (26). The endogenous colocalization of p65 and PML was determined by double-color immunofluorescence staining. Briefly, U2OS and SiHa cells were cultured on 35-mm diameter coverslips, fixed with TNFα (10 ng/ml) and induced with interferon α for 24 h. Immunofluorescence staining was performed using anti-PML mAb (PG-M3, Santa Cruz Biotechnology), anti-p65 polyclonal antibody, and secondary antibodies. Images were captured with a Zeiss laser scan confocal microscope (LSM 5).

Electrophoretic Mobility Shift Assay—The in vitro translated PML or p65 proteins were synthesized by the TNT-coupled wheat germ translation system (Promega Corp.). Nuclear extracts were prepared from U2OS stable cell lines or cells treated for 1 h with TNF (20 ng/ml). The NF-κB probe was prepared by annealing the complementary oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGG), and the 3′-recessive ends were labeled with Klenow fragment fill-in reaction. Binding reactions contained 5 μg of nuclear extracts, 1 μg of poly(dI-dC), 1 ng of NF-κB probe (1 × 10⁶ cpm) in 20 μl of KCl binding buffer (10% glycerol, 1 mM EDTA, 20 μM Tris-HCl, pH 8.0, and 5 mM KCl). The reaction was incubated for 20 min at room temperature and then resolved in a 5% polyacrylamide gel in Tris glycine buffer (50 mM Tris, 2 mM glycine, 2 mM EDTA, pH 8.3). For competition or supershift assays, 50 ng of unlabeled probe or 1 μg of anti-p65 mAb was added to the binding reactions, respectively.

RESULTS

PML Sensitizes Cells to Tumor Necrosis Factor α-Induced Apoptosis—It is well documented by animal and cell culture models that PML is a tumor/growth suppressor. To understand further the mechanism of the growth-suppressing function of PML, we generated stable PML clones in U2OS, a human osteogenic sarcoma cell line. In these cells, expression of PML is driven by the metallothionein promoter, inducible by Cd2⁺ or Zn2⁺. U2OS cells transfected with vector alone were used as a control. The colony growth of these cells was significantly inhibited when PML expression was induced by Cd2⁺ but not in the control cells (Fig. 1a and b), demonstrating the growth-suppressing property of PML in U2OS. The inducible expression level of PML in this cell line is comparable with that in SiHa cells after induction with IFNα (Fig. 1a).

U2OS is resistant to low dose treatment with TNFα (27), but apoptosis can be induced by TNFα in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1c). This indicates that the TNF receptor-mediated apoptotic pathway is intact in this cell line. We next investigated whether the induction of PML expression by Cd2⁺ or phosphate-buffered saline (PBS) was added, and the culture was continued for 10 days.

Subcellular Fractionation of Cytoplasmic and Nuclear Proteins—Cytoplasmic protein was prepared by the digitonin extraction method. Cultured cells were washed twice with cold PBS and resuspended in ice-cold digitonin extraction buffer (10 mM Tris, 0.4 M glycine, 2 mM EDTA, 0.1% Triton X-100, 300 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Cells were permeabilized for 10 min and analyzed by trypan blue exclusion assay and then centrifuged for 5 min (480 × g) at 4 °C. The supernatant contained the cytoplasmic protein. The digestion-in-
cells. To examine whether PML/TNFα-induced apoptosis is associated with DNA fragmentation, U2OS cells were infected with Ad-PML or the control adenovirus Ad-C for 8 h and then treated with TNFα for an additional 16 h. Cell death was examined by TUNEL assay. As expected, PML expression alone was insufficient to induce cell death in 24 h; a combination of PML and TNFα, however, induced a massive DNA fragmentation (Fig. 2a), suggesting that caspase-activated DNase (CAD) was activated during PML/TNFα-induced cell death. We next sought to determine whether similar effects could be achieved in other cell lines. We found that PML expression dramatically sensitized TNFα-induced apoptosis in Saos2, HT1080, and 293T cell lines (data not shown).

Bcl-2, Bcl-xL, and Bax are important antiapoptotic or proapoptotic proteins that control the mitochondria-dependent apoptotic pathway (28–32). We evaluated whether PML regulates the expression of these proteins in U2OS cells. Our results showed that ectopic expression of PML had no effect on these proteins (data not shown). This result is in agreement with a previous report (10) that deletion of the PML gene did not affect the expression of the Bcl2 family of proteins.

**Activation of Initiator/Effecter Caspases and CAD Is Associated with PML/TNFα-induced Apoptosis**—The results of our study suggest that the death receptor signaling pathway is involved in PML/TNFα-induced cell death. To confirm this notion, we evaluated whether PML/TNFα-induced cell death involves the activation of initiator/effecter caspases and CAD, which are crucial players in apoptosis for almost all cell types. It has been well documented that upon ligand binding, TNF receptors recruit pro-caspase-8 via the adaptor protein FADD and subsequently cleave effecter caspases such as caspase-3, -6, and -7. This activates CAD by degrading the inhibitor of CAD (ICAD). Consequently, DNA fragmentation and apoptosis occur (33). These active executioners also cleave other cellular substrates that are essential for cell survival and responsible for the morphologic and biochemical features of apoptosis.

To determine which CAD is activated in PML-induced and PML/TNFα-induced cell death, we infected the U2OS cells with Ad-PML or Ad-C for 8 h. TNFα was then added, and the cells were incubated for an additional 16 h. Cell death was measured by trypan blue exclusion assay. PML sensitizes TNFα-induced apoptosis in U2OS cells. PML/U2OS or pMEP4/U2OS was induced for 8 h with 5 μM CdSO₄ and the cells were then treated or untreated with TNFα (10 ng/ml) for 24 h. Cell death was quantified by cell-death detection ELISA assay (Roche Diagnostics).

**PML Induces Cell Death through the Death Receptor-mediated Pathway**—The binding of TNFα with death receptors activates caspase-8 and processes effector caspases, including caspase-3, -6, and -7. Bcl-2 cannot rescue these cells from TNFα-induced apoptosis in most cell lines. Active caspase-8 can also process the proapoptotic Bcl-2 family member Bid (34) that contains only BH3. Truncated Bid translocates to mitochondria and induces the loss of mitochondria membrane potential (Δψₘ) and releases cytochrome c from the mitochondrial intermembrane space to the cytosol. The released cytochrome c binds to Apaf1 and then recruits and processes pro-caspase-9.
and initiates the downstream caspase cascade (35). Therefore, the caspase-8-mediated pathway can use mitochondria to activate the executioner apoptosis caspase cascade (36). In contrast, the apoptosis initiated by the mitochondria-dependent apoptosis pathway can be inhibited by Bcl-2/Bcl-XL, which blocks cytochrome $c$ release (30).

Death receptor-mediated apoptosis is a very complex process and may involve the mitochondria-dependent pathway in most cells. Recently, several viral and cellular inhibitors of apoptosis have been found that block or halt apoptotic signaling at defined points of the apoptotic pathways. Some examples are as follows: CrmA (the product of cowpox virus cytokine response modifier A) (37), which is a powerful specific inhibitor of caspase-8; p35 (the 35-kDa protein of baculovirus AcMNPV) (38), which has a broad specificity but is a less powerful inhibitor of caspases; c-FLIP (the cellular FLICE-inhibitory protein) (39), a specific inhibitor of caspase-8; and Bcl-2, a cellular inhibitor of the mitochondria-dependent apoptotic pathway that acts by preventing cytochrome $c$ release. To determine the apoptotic pathways initiated by PML/TNF$\alpha$-induced apoptosis, we assessed the ability of these apoptosis inhibitors to block PML/TNF$\alpha$-induced apoptosis.

Stable clones of U2OS cells that conditionally expressed CrmA (CrmA/U2OS), p35 (p35/U2OS), c-FLIP (c-FLIP/U2OS), and Bcl-2 (Bcl-2/U2OS) were established for this study. CrmA and p35 substantially suppressed PML/TNF$\alpha$-induced cell death within 24 h (Fig. 3a). CrmA exerted a much stronger inhibitory effect than did p35. Cellular FLICE-inhibitory protein c-FLIP, which blocks TNF receptor-mediated apoptosis, also inhibits PML/TNF$\alpha$-induced cell death (Fig. 3b). In a similar experiment, Bcl-2 did not inhibit PML/TNF$\alpha$-induced cell death, although it retained its ability to block cytochrome $c$ release from the mitochondria (Fig. 3c). It is interesting to note that Bcl-2 did not affect pro-caspase-8 processing. This finding is in agreement with the previous reports (40) that showed that Bcl-2 could not block TNF$\alpha$-induced apoptosis in some types of cells. Together, these results strongly support the idea that PML/TNF$\alpha$-induced cell death depends upon the death receptor-mediated pathway.

**PML Sensitizes TNF$\alpha$-induced Apoptosis by Acting as a Functional Inhibitor of NF-kB**—The TNF$\alpha$ signaling events activate the proapoptotic pathway and the antiapoptotic pathways through the activation of caspase-8 and NF-kB transcriptional function, respectively (41). NF-kB consists of two sub-
units (p65 and p50 or p52) localized in the cytoplasm to form an inactive complex with an inhibitor of NF-κB (IκB) (41). TNFα induces IκB kinase activation that leads to phosphorylation and subsequently degradation of IκB by proteosome. NF-κB then enters the nucleus and activates the transcription of various target genes, including those encoding the antiapoptotic proteins (22, 42). Therefore, it is possible that PML sensitizes TNFα-induced cell death by interfering with NF-κB signaling.

To determine whether PML attenuates signaling of NF-κB when cells are treated with TNFα, we cotransfected an NF-κB-dependent luciferase reporter with the PML expression plasmid. PML dramatically repressed the transactivation of NF-κB induced by TNFα (Fig. 4a). This finding suggests that PML is a negative regulator of the NF-κB signaling pathway.

We next evaluated whether PML is a transcriptional repressor of NF-κB activity. A series of cotransfection experiments was performed using RelA (p65), PML expression plasmids, and the NF-κB reporter construct. This study demonstrated that PML significantly repressed the transcriptional activity of RelA (p65) (Fig. 4b). In addition, PML inhibited RelA-mediated transcription in a dose-dependent manner (Fig. 4c). In a similar experiment, we found that cotransfection of PML up-regulates c-Myc-mediated transactivation, indicating that the repression effect of PML on p65-mediated transcription is specific (Fig. 4d). These results suggest that NF-κB is a direct target of PML. Because PML does not bind DNA, one possible explanation for such inhibitory effect is that PML interacts with RelA and interferes with its binding to the promoter of target genes. Electrophoretic mobility shift assay demonstrated that PML significantly inhibited RelA binding to its consensus enhancer sequence when the in vitro translated proteins (Fig. 5a) or the nuclear extracts were used in the assay (Fig. 5b). To determine whether PML could stabilize IκB or affect the expression of RelA/p65 during TNFα-triggered signaling, Western blotting was performed using total protein isolated from PML/U2OS and pMEP4/U2OS induced with TNFα. This study showed that PML had little effect on the stability of IκB and expression of RelA/p65 (Fig. 5c).

**In Vivo Association of PML and RelA/p65**—To investigate whether PML and RelA/p65 are associated in vivo, we first performed commuoprecipitation experiments using total cell extracts isolated from cells cotransfected with PML and RelA expression plasmids. PML was coimmunoprecipitated and associated with RelA in vivo (Fig. 6a). Our study further showed that the endogenous RelA and PML were commumoprecipitated from the nuclear extracts isolated from cells induced by interferon-α and pretreated with TNFα (Fig. 6b). These results strongly suggest that the two proteins are associated in vivo.

Because PML assembles NB by recruiting other factors in the cells, our results suggest that PML may recruit RelA into

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**Fig. 3.** PML potentiates cell death through the death receptor-mediated pathway. *a,* viral inhibitors of apoptosis inhibit PML/TNFα-induced cell death. Inducible stable lines were induced for 12 h with CdSO4 (5 μM) and then infected with Ad-PML. After 8 h, infected cells were treated with TNFα (10 ng/ml) for 16 h. Cells were then harvested and analyzed for cell death by ELISA as described above. *b,* PML/TNFα-induced cell death can be inhibited by c-FLIPs but not Bcl-2. Inducible and stable cell lines expressing c-FLIPs and Bcl-2 were treated as described in *a* before analysis of cell death. *c,* Bcl-2 blocked cytochrome c released from the mitochondria but not caspase-8 processing and cell death induced by PML/TNFα. The indicated inducible-stable cell lines were treated as described in *a.* Cell death was determined by trypan blue exclusion assay. The cytosolic and total proteins were extracted and resolved in a 10% SDS-PAGE. Western blot analysis of the cytosolic protein was performed with the antibodies against cytochrome c, caspase-8, and α-tubulin. Western blot analysis of total proteins was performed using the rabbit anti-PML antibody.
To evaluate this possibility, we performed immunofluorescent staining and confocal microscopy of U2OS cells cotransfected with the expression plasmids of PML or PML mutant and RelA. This study demonstrated that RelA was indeed recruited to the PML NB in vivo in the cotransfection experiment (Fig. 6c). The mutant PML(1–555), however,
cannot relocate RelA into the PML NB, and the negative control LacZ was not recruited into PML NB indicating that RelA/p65 targeting into the PML NB should be specific. These results support the idea that PML and RelA may be associated in the PML NB in vivo and that the C terminus of PML is required for such association. Since RelA/p65 mainly localizes to the cytoplasm before treatment with TNFα or other stimuli, it is important to examine whether PML can also recruit the endogenous RelA/p65 to the PML NB when RelA/p65 is translocated into the nuclei after TNFα treatment. To evaluate this possibility, SiHa and U2OS cells were pretreated with leptomycin B, which has been shown to retain RelA/p65 in the nuclei, and then were treated with TNFα and interferon. Double-color immunofluorescent staining and confocal microscopy detected colocalization of the endogenous RelA/p65 and PML in the PML NB (Fig. 6d) in both the SiHa and U2OS cells. These results strongly support the idea that PML and RelA/p65 are functionally associated in vivo.

The C Terminus of PML Is Indispensable for Inhibition of NF-κB—Our study demonstrated that PML functionally represses RelA/p65 by recruiting it to the PML NB and interfering with binding of NF-κB to its enhancer. We next attempted to identify which domain of PML is required to repress NF-κB transactivation. By using several PML mutants in a series of cotransfection experiments with the NF-κB reporter construct, we found that PML mutants with a deletion of the RING region were capable of inhibiting NF-κB transactivation, whereas PML mutants lacking amino acids 555–633 (PML(1–555)) and 305–633 (PML(1–305)) were not. This result indicated the C terminus of PML is essential for PML to fully inhibit NF-κB (Fig. 7a). Interestingly, a previous report showed that the C terminus of PML is required for interactions with p53 and relocation of p53 to the PML NB (62). To determine whether the ability of the PML to inhibit NF-κB is required for the proapoptotic function of PML, a stable cell line expressing PML(1–555) was established in U2OS cells. We compared the sensitivity to TNFα treatment of this stable cell line with that of the wild-type PML. Our results demonstrated that the C terminus of PML is required for sensitizing TNFα-induced apoptosis (Fig. 7b). Result presented in Fig. 6c showed that PML(1–555) failed to recruit p65 to the PML NBs. We next performed coimmunoprecipitation assay to investigate whether PML(1–555) physically interacts with p65. This study indeed demonstrated that PML(1–555) was unable to coimmunoprecipitate p65 (Fig. 7c). Taken together, these results demonstrated that the C terminus of PML (amino acids 556–633) is responsible for inhibiting NF-κB transactivation, recruiting NF-κB to the PML NB, and enhancing TNFα-induced apoptosis.

RelA Overexpression Blocked PML/TNFα-induced Apoptosis—If PML sensitizes U2OS cells to TNFα-induced apoptosis by inhibiting the NF-κB-mediated survival pathway as our studies suggest, then ectopic expression of RelA/p65 should block PML/TNFα-induced apoptosis. To test this hypothesis, a RelA-inducible stable cell line was established in U2OS cells and was infected with Ad-PML or Ad-C in the presence or absence of CdSO4 and then treated with TNFα. Apoptosis was then quantified by a DNA fragmentation assay. The result of this study demonstrated that overexpression of RelA significantly reduced PML/TNFα-induced apoptosis (Fig. 8). This
PML/TNF\textsuperscript{-}/H9251-induced cell death was associated with DFF/ICAD degradation. These results demonstrated that PML is a functional inhibitor of RelA/p65.

Loss of PML Function Renders Cells Resistant to TNF\textsuperscript{-}/H9251-induced Apoptosis—We next sought to examine the effects of endogenous PML on TNF\textsuperscript{-}/H9251-induced signaling events in PML\textsuperscript{-}/H11001\textsuperscript/- and PML\textsuperscript{-}/H11002\textsuperscript/- MEFs. A culture medium with low growth and low survival factors was selected to sensitize the wild-type MEFs to apoptosis in response to TNF\textsuperscript{-}/H9251 treatment. MEFs derived from PML\textsuperscript{-}/H11001 and PML\textsuperscript{-}/H11002 mice were tested for their relative sensitivity to TNF\textsuperscript{-}/H9251 under similar conditions. PML\textsuperscript{-}/H11001 and PML\textsuperscript{-}/H11002 MEFs exhibited similar survival rates, but the PML\textsuperscript{-}/H11002 MEFs were significantly more resistant than the PML\textsuperscript{-}/H11001 MEFs to TNF\textsuperscript{-}/H9251-induced apoptosis (Fig. 9). This experiment was repeated, and similar results were observed. This finding implies that a loss of PML function rendered cells resistant to TNF\textsuperscript{-}/H9251-induced apoptosis.

To provide further support of this finding, we investigated how PML expression affected sensitivity to TNF\textsuperscript{-}/H9251-mediated cell death. PML\textsuperscript{+/+} MEFs and PML\textsuperscript{-/-} MEFs were infected with Ad-PML and the control Ad-C. The results of this study showed that reintroduction of PML into the PML\textsuperscript{-/-} MEFs restored sensitivity to TNF\textsuperscript{-}/H9251-induced cell death (Fig. 9b). We next examined whether TNF\textsuperscript{a} increases activity of NF-\kappaB transactivation in PML-deficient cells in a transient transfection assay. The results demonstrated a moderate but consistent increase in reporter activity in the PML\textsuperscript{-/-} MEFs (Fig. 9c).

DISCUSSION

This study shows that PML sensitizes cells to TNF\textsuperscript{-}/H9251-induced apoptosis in U2OS and several other cell lines through the death receptor-dependent apoptotic pathway. The C terminus of PML is indispensable for the proapoptotic functions of the PML. The proapoptotic function of PML is p53-independent, as shown by the effect of the PML on the p53-negative Saos-2 cell line. By using the TNF\textsuperscript{-}/H9251-resistant U2OS cell line as a model, we showed that PML sensitizes TNF\textsuperscript{-}/H9251-induced cell death by...
activated pathway by regulating the NF-κB-mediated apoptosis could be induced through the death receptor-mediated pathway and contributing to tumorigenesis (44). We examined whether cancers, serving as a mechanism to prevent cancer cell death /H9260 inhibiting the NF-κB-mediated apoptosis.

The MEFs and PML−/− MEF were infected with Ad-PML and Ad-C in the presence or absence of TNFs. Cell death was quantified as described in a. c, promoter activity of NF-κB in PML+/+ MEF and PML−/− MEF. The NF-κB reporter plasmid (NF-κB-TATA-Luc) was transfected into the PML+/+ MEF and PML−/− MEF. Cells were cultured for 18 h and then treated with 10 ng/ml of TNFs for 4 h. Total proteins were isolated, and luciferase activity was determined as described in Fig. 4 legend.

NF-κB activation can be found in many different types of cancers, serving as a mechanism to prevent cancer cell death and contributing to tumorigenesis (44). We examined whether apoptosis could be induced through the death receptor-mediated pathway by regulating the NF-κB survival pathway. Our results (Figs. 4 and 5) demonstrated that PML represses the transactivation function of NF-κB by interacting with p65/RelA and preventing it from binding to the NF-κB recognition sequence. This result is in agreement with our previous report (45) that demonstrated that PML repressed A-20-mediated transcription, a target gene of NF-κB, through the NF-κB-binding site. Commmunoprecipitation of PML and p65/RelA was found at the endogenous levels and supports an in vivo association between the two proteins. This notion was further supported by the finding that PML colocalizes with p65/RelA at the endogenous levels in both U2OS and SiHa cells. Our results further showing that stable overexpression of RelA inhibits PMLTNFα-induced apoptosis (Fig. 8). Together, these studies demonstrated that PML sensitizes TNFα-induced apoptosis by inhibiting the NF-κB survival pathway. Further support for this conclusion was obtained using the PML knockout MEFs in a TNFα-induced cell death assay. This study showed that PML deficient cells are more resistant to TNFα-induced apoptosis than are normal MEFs and that TNFα sensitivity in these cells can be restored when PML expression is reintroduced by adenovirus-mediated gene transfer.

It is clear that c-FLIP specifically inhibits caspase 8, an upstream initiator of the TNF-induced death receptor-mediated apoptosis. Recent findings (46) demonstrated that c-FLIP is a target gene of NF-κB and that restoration of FLIP in NF-κB-null cells efficiently inhibits TNF- and FasL-induced apoptosis. This finding, together with our results presented here, demonstrates that PML sensitizes TNFα-induced apoptosis by inhibiting the NF-κB transactivation function. Down-regulation of NF-κB transactivation relieves c-FLIP inhibition of caspase-8, leading to the activation of this apoptosis initiator and downstream effector caspases. At the same time, other target genes of NF-κB, including several of the inhibitor of
apoptotic proteins, e.g. IAPs, will also be down-regulated, resulting in further activation of effector caspases. Together, these events weaken the NF-κB survival pathway and trigger apoptotic cell death (see Fig. 10). Our studies that overexpression of Rel A and c-FLIP inhibits PML/TNFα-induced apoptosis (Fig. 3b and 8) support this hypothesis.

NF-κB plays a central role in host defense and inflammatory responses, and its activity can be activated rapidly by many proinflammatory agents, including cytokines and virus (47). NF-κB regulates a wide variety of genes, including many of the genes encoding cytokines, chemokines, and adhesion molecules (47–49). Many of the apoptosis inhibitory proteins, including IAPs, A1, A20, Bel-2, FasL, TRAFs, and c-FLIP, are also targets of NF-κB (48, 51). An important role of NF-κB in apoptosis was first demonstrated by Beg et al. (49), who showed that p65/RelA-deficient mice died in the embryo from extensive liver apoptosis at E15. NF-κB activity is normally controlled by IκB, a cytoplasmic protein that forms an inactive complex with NF-κB and inhibits NF-κB activity by preventing it from entering the nucleus. Our study shows that PML, a nuclear protein, also inhibits NF-κB activity. Thus NF-κB-mediated signaling could be regulated in both the cytoplasm and the nucleus. In addition, TAF110 is a nuclear transcriptional coactivator of NF-κB essential for induction of antiapoptotic proteins in response to TNFα (52). A dominant-negative TAF110 blocked the interaction between NF-κB and TAF110 and severely reduced cell survival in response to TNFα (53). Another IFN-inducible protein, p202 (50), was shown to inhibit NF-κB activity in the nucleus and TNFα-induced sensitized cell death through a mechanism similar to that utilized by PML.

PML is involved in viral DNA replication, and it appears that the PML NB-associated proteins are released to viral replication and transcription domains. The two early transcribed adenovirus proteins E4-open reading frame 3 and E1B (54, 55) are targeted to the PML NB and trigger its dissociation from other cellular factors or the release of some important factors that are required for viral propagation and the prevention of apoptosis of host cells. The inhibition of the PML NB dissociation reduces adenovirus replication, supporting the idea that the PML NB retains cellular factors that play critical roles in viral replication, regulation of viral transcription, and host-cell survival. It is well documented that NF-κB is frequently activated by viral infection and plays a critical role in viral oncogenesis and the regulation of viral gene transcription (56). Our finding that PML represses NF-κB (p65) transcriptional activity may help explain why the PML NB is the target of several virus proteins. The PML NB has been shown to be the target of adenovirus, human T-cell leukemia virus type 1 (57), papillomavirus (58), hepatitis virus (59), herpes simplex virus (60), and human cytomegalovirus (61).

Other mechanisms of PML-induced apoptosis have also been reported. A role of PML in p53-dependent apoptosis in thymocytes has been reported recently (62) in response to ionization radiation. This pathway involves activation of the p53 downstream target genes bax and p21. Another pathway of PML-induced apoptosis was also reported recently. This pathway is induced in response to Fas in B and T splenocytes in a p53-independent manner through a mechanism involve the in vitro association between PML and the proapoptotic protein Daxx (9, 63). Daxx was originally identified as a Fas death domain binding protein (64); it also directly interacts with PML and colocalizes in the PML NBs. Daxx regulation of Fas-induced apoptosis required the ability of Daxx to colocalize to the PML NBs (65). Our study here shows that PML/TNFα-induced apoptosis is a mechanism independent from the p53 function. It is not clear at this stage whether this mechanism is in anyway connected with the Fas/Daxx-mediated apoptotic pathway. It has been shown that Daxx acts as a transcriptional repressor by recruiting histone deacetylases. The SUMO-1-modified PML negatively regulates transcriptional repression of Daxx by interacting and sequestering Daxx to the PML NBs (17, 18). Although the detailed mechanism of Fas/Daxx-induced apoptosis remains unclear, it is possible that PML regulates this pathway through a direct interaction with Daxx. PML and its associated proteins play a critical role in the control of cell growth, although the molecular mechanism is not clear. Recent findings (66, 67) demonstrated that p53 was recruited to the PML NB through a direct interaction between the core domain of p53 and the C terminus of PML, resulting in enhanced transactivation of p53 in a promoter-specific manner and affecting cell survival. This study implies that the PML NB is required but alone is insufficient to enhance p53-induced apoptosis. Although it lacks the C terminus, PML(1–555) forms a nuclear body (67) but cannot enhance p53-mediated cell death. Interestingly, the C terminus of PMLIV isoform is also essential for the interaction with p65 (Fig. 6c and 7c). More important, the PML mutant lacking the C terminus could not fully inhibit p65 activity and lost its ability to enhance apoptosis in response to TNFα. Various PML isoforms have been found that share the same N terminus with variable C termini generated by alternative splicing (68). We speculate that the various functions of PML may be regulated through alternative splicing to produce various PML isoforms with different cellular functions. Our previous study (26) demonstrated that only a specific isoform of PML interacts with histone deacetylases for transcriptional repression. There is also evidence that only some PML isoforms interact with retinoblastoma protein. It is therefore important in the future to study how PML regulates transcription, cell growth, and apoptosis through specific isoforms.

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