The promyelocytic leukemia (PML) protein is a tumor suppressor that is disrupted by the chromosomal translocation t(15;17), a consistent cytogenetic feature of acute promyelocytic leukemia. A role of PML in multiple pathways of apoptosis was conclusively demonstrated using PML−/− animal and cell culture models. In a previous study, we found that PML sensitizes tumor necrosis factor-induced apoptosis in tumor necrosis factor (TNF)-resistant U2OS cells. This finding helped to explain the mechanism of PML-induced apoptosis. The zinc finger protein A20 is a target gene of NFκB inducible by TNFα, and it is a potent inhibitor of TNF-induced apoptosis. In this study, we demonstrated that PML is a transcriptional repressor of the A20 promoter and that PML represses A20 expression induced by TNFα. We showed that PML inhibits A20 transactivation through the NFκB site by interfering with its binding to the promoter. We also showed that stable overexpression of A20 inhibits apoptosis and caspase activation induced by PML/TNFα. The results of this study suggest that A20 is a downstream target of PML-induced apoptosis and supports a role of A20 in modulating cell death induced by PML/TNFα in TNF-resistant cells.

The disruption of the promyelocytic leukemia (PML) gene by the t(15;17) chromosomal translocation is a hallmark of acute promyelocytic leukemia. PML is a multifunctional Ring finger protein normally localized within the nucleus as a macromolecular structure in a nuclear speckled pattern designated PML nuclear body (NB) or PML oncogenic domain (2). In APL cells, the normal PML NBs are disrupted as a result of forming heterodimers with the fusion protein PML-retinoic acid receptor α (3). This finding helped to explain the mechanism of PML-induced apoptosis. The zinc finger protein A20 is a target gene of NFκB inducible by TNFα, and it is a potent inhibitor of TNF-induced apoptosis. In this study, we demonstrated that PML is a transcriptional repressor of the A20 promoter and that PML represses A20 expression induced by TNFα. We showed that PML inhibits A20 transactivation through the NFκB site by interfering with its binding to the promoter. We also showed that stable overexpression of A20 inhibits apoptosis and caspase activation induced by PML/TNFα. The results of this study suggest that A20 is a downstream target of PML-induced apoptosis and supports a role of A20 in modulating cell death induced by PML/TNFα in TNF-resistant cells.

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The disruption of the promyelocytic leukemia (PML) gene by the t(15;17) chromosomal translocation is a hallmark of acute promyelocytic leukemia. PML is a multifunctional Ring finger protein normally localized within the nucleus as a macromolecular structure in a nuclear speckled pattern designated PML nuclear body (NB) or PML oncogenic domain (2). In APL cells, the normal PML NBs are disrupted as a result of forming heterodimers with the fusion protein PML-retinoic acid receptor α (3). This finding helped to explain the mechanism of PML-induced apoptosis. The zinc finger protein A20 is a target gene of NFκB inducible by TNFα, and it is a potent inhibitor of TNF-induced apoptosis. In this study, we demonstrated that PML is a transcriptional repressor of the A20 promoter and that PML represses A20 expression induced by TNFα. We showed that PML inhibits A20 transactivation through the NFκB site by interfering with its binding to the promoter. We also showed that stable overexpression of A20 inhibits apoptosis and caspase activation induced by PML/TNFα. The results of this study suggest that A20 is a downstream target of PML-induced apoptosis and supports a role of A20 in modulating cell death induced by PML/TNFα in TNF-resistant cells.
To further understand the mechanism of how PML induces apoptosis, our present study shows that PML significantly inhibits the expression of A20, a potent repressor of TNF-induced apoptosis. PML represses the A20 promoter induced by TNFα and phorbol 12-myristate 13-acetate (PMA) through the NFκB site. Our study further shows that A20 inhibits apoptosis induced by PML/TNFα and explains the mechanism by which PML induces apoptosis by sensitizing the TNF death receptor pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmids Construction**—The inducible expression plasmid pMEP4-PML was constructed by subcloning the full-length PML cDNA (PML3) into the NotI/XhoI sites of the pMEP4 vector (Invitrogen). pMEP4/HA-A20 was constructed by cloning the polymerase chain reaction-amplified full-length A20 cDNA into the EcoRI site of the pCrUZ/HA vector to generate pCrUZ/HA-A20. The HA-A20 fragment was then excised by XhoI/BglII and subcloned into the pMEP4 vector. The A20 promoter-luciferase reporter construct A20PR-Luc was generated by cloning the A20 promoter (1500 bp) into the XhoI and BglII sites of the pCruz/HA vector to generate pCruz/HA-A20. The HA-A20 fragment was then excised by XhoI/BglII and subcloned into the pMEP4 vector.

**Cell Culture and Reagents**—The U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Anti-HA monoclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-caspase-7 antibody (number 66871A) was purchased from PharMingen. Anti-A20 antibody (number H11032) was obtained from Active & Motif, Inc. (Carlsbad, CA).

**Transfection and Luciferase Reporter Assay**—Cells were cultured to confluence and transfected with the expression plasmids using FuGENE 6 transfection reagent (Roche Diagnostics). Luciferase activity was determined using the luciferase reporter assay according to the manufacturer’s protocol (Promega).

**Generation of Stable Cell Lines**—U2OS cells were transfected with each of the following plasmids: pMEP4 (negative control), pMEP4/HA-A20, and pMEP4/PML with FuGENE 6 reagent. Cells were then selected with hygromycin (200 μg/ml) for 10 days. Pools of hygromycin-resistant stable clones were selected. Inducible expression of the respective proteins in these stable cell lines was determined by induction with CdSO4 (5 μM) for 20 h followed by immunofluorescent staining and Western blot analysis. Cell death was examined by trypan blue exclusion assay.

**RNA Preparation and cDNA Synthesis**—Total RNA was prepared from cells using RNeasy mini-kit (Qiagen). cDNA was synthesized from 4 μg of total RNA using the Superscript premplification system obtained from Invitrogen.

**Preparation of Nuclear Proteins**—Cultured cells were washed twice with cold phosphate-buffered saline and resuspended in ice-cold digitonin extraction buffer (10 mM PIPES, pH 6.5, 0.015% (w/v) digitonin, 300 mM NaCl, 3 mM MgCl2, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). Cells were permeabilized for 10 min, assessed by trypan blue exclusion assay, and centrifuged for 5 min at 480 × g at 4 °C. The supernatant contains the cytoplasmic protein. The digitonin-insoluble pellet was resuspended in ice-cold Triton X-100 extraction buffer (10 mM PIPES, pH 7.4, 0.5% (v/v) Triton X-100, 300 mM sucrose, 1 mM NaCl, 3 mM MgCl2, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. The nuclei were pelleted by centrifugation for 10 min at 5000 × g and resuspended in nuclear extraction buffer (50 mM PIPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Nonidet P-40, and 10% (v/v) glycerol). The nuclear mixture was incubated for 30 min on ice and then centrifuged for 5 min (6700 × g) at 4 °C. The supernatant contains the nuclear proteins.

**Electrophoretic Mobility Shift Assay**—The in vitro-translated PML or p65 proteins were synthesized by the Tnt-coupled wheat germ translation system (Promega). Nuclear extracts were prepared from U2OS stable cell lines or cells treated with TNFα (20 ng/ml) for 1 h. The A20 promoter NFκB probe was prepared by annealing the oligonucleotides 5′-GACTTTGGGAAATCCGCTCTGCTTGGGCCC-3′ and 5′-GCCCGGGATTTCGCAGGACATCTTCC-3′ and inserting them into the pGL3 vector (Promega, Madison, WI). Correct DNA sequence of all plasmids was confirmed by direct DNA sequencing.

**RESULTS**

**PML Inhibits Uregulation of A20 Induced by TNFα**—Our preliminary study showed that PML significantly sensitized TNF-induced apoptosis in the TNF-resistant cell line U2O. To understand the effects of PML on the TNF-induced apoptotic pathway, we investigated the effect of PML on the expression of A20 in the osteosarcoma cell line U2OS. We found that TNFα induces a significant increase in A1 and A20 expression in U2OS cells (U2OS/PML) and the control U2OS cells (pMEP4/U2OS) was established. These cells were treated with 5 μM cadmium sulfate to induce PML expression followed by the treatment of TNFα. The results of this study showed that expression of PML significantly repressed the expression of A20 induced by TNFα (Fig. 1b). No such effect on A20 expres-
PML Represses Transactivation of A20

PML Inhibits Transcriptional Activation of the A20 Promoter—A20 is a downstream target gene of NFκB inducible by TNFα treatment. Therefore, we speculate that PML represses transcription of A20 through the inhibition of the NFκB function. To test this hypothesis, a series of cotransfection experiments was performed with pA20PR-Luc in the presence of RelA/p65 expression plasmid and increasing concentrations of pcDNA/PML. The results presented in Fig. 3 demonstrate that the presence of RelA/p65 dramatically activated the promoter activity of A20. Cotransfection with pcDNA3/PML at increasing concentrations significantly repressed A20 promoter activity in a dose-dependent manner. This result indicates that PML represses the RelA/p65-dependent transactivation of the A20 promoter.

PML Interferes with NFκB Binding to the A20 Promoter—The study described above suggests that PML represses RelA-dependent transactivation by interfering with NFκB binding to the A20 promoter. To further understand how PML represses transactivation of the A20 promoter through NFκB, electrophoretic mobility shift assay was performed using nuclear protein isolated from stable inducible cell lines pMEP4/U2OS and PML/U2OS treated or untreated with TNFα. In this assay, electrophoretic mobility shift assay was performed using the DNA sequence derived from the NFκB site of the A20 promoter. The results presented in Fig. 4 demonstrate that the expression of PML inhibited the binding of p65/RelA to the consensus sequences (lanes 5 and 6). Together, these studies suggest that PML inhibits NFκB binding to the promoter of A20 and consequently represses its transactivation.

Overexpression of A20 Inhibits Apoptosis Induced by PML/TNFα—It is clear that A20 is a target gene of NFκB inducible by TNFα (25). There is also an abundance of evidence to suggest that A20 acts as a potent inhibitor of NFκB (27–29). This raises the possibility that A20 plays an important role as a negative feedback regulator of NFκB functions. To investigate the possible functional interaction between PML and A20, we investigated whether the expression of A20 has any significant effects on PML/TNFα-induced apoptosis in U2OS cells. To address this question, a pool of inducible stable cell lines of A20 in U2OS cells driven by the metallothionine promoter was established. The expression of A20 can be induced by a low concentration of Cd²⁺ (Fig. 5a). A control cell line (pMEP4/U2OS) transfected with the empty vector alone was also established. The results presented in Fig. 5b show that a significant degree of cell death was induced after a 24-h infection with recombinant PML adenovirus (Ad-PML) and 8-h treatment with TNFα. However, PML/TNFα-induced cell death was significantly reduced in cells overexpressing A20, indicating that A20 inhibited apoptosis induced by PML/TNFα. The results shown in Fig. 5c demonstrate that control pMEP4/U2OS cells treated with PML/TNFα significantly activated procaspase-7; however, in cells overexpressing A20, procaspase-7 activation was significantly reduced. Thus, this study demonstrated that A20 inhibits cell death induced by PML/TNFα in TNF-resistant cells.

DISCUSSION

PML was originally cloned and characterized for its involvement in the nonrandom chromosomal translocation t(15;17), which occurs in ~99% of APL cases. The importance of PML in maintaining normal cellular function was unambiguously demonstrated by its essential role in multiple pathways of apoptosis (4, 5) and cell cycle progression (3, 6, 7). PML and PML NBs are induced by interferons and inflammatory stimuli (31–33), indicating that they have a role in response to inflammation and viral infection. PML is a cell growth and tumor suppressor; however, the mechanisms by which PML exerts its tumor sup-

Fig. 2. PML inhibits A20 promoter activity induced by TNFα and PMA. a, U2OS cells were cotransfected with A20PR-Luc reporter (0.2 µg) and pcDNA3/PML (0.8 µg) or pcDNA3 (0.58 µg) in 12-well plates. After 24 h, cells were treated with 20 ng/ml TNFα for the indicated times. Total proteins were isolated, and luciferase activity was determined using the luciferase assay kit (Promega). b, pMEP4/U2OS and pMEP4/U2OS stable cells induced or uninduced with 5 µM Cd²⁺ were transfected with A20PR-Luc reporter plasmid. After 24 h, cells were treated with 10 ng/ml PMA for the indicated times. Luciferase activity was determined as described above. The expression plasmid pCMVβ-galactosidase was included in each transfection assay to normalize transfection efficiencies.
pressor function remains unclear. In MCF-7 cells, the overexpression of PML induced G1 cell-cycle arrest associated with an upregulation of p21, p53, cyclin D, and hypophosphorylation of the Rb (6). Unphosphorylated Rb binds the E2F transcription factor and limits its ability to activate the transcription of genes essential for the G1 to S transition (34).

PML does not bind DNA directly; however, it plays a role in transcriptional regulation through its association with the transcription coactivator CREB-binding protein and interacts with the transcription corepressor histone deacetylases (13, 14, 22). PML activates transcription by binding and sequestering the negative regulator, Daxx (35–37), interacting with p53 (18–20) and recruiting p53 to the PML NB to enhance transactivation of p53 target genes. PML also represses transcription by interacting with transcription factor Sp1 and interfering with its binding to the epidermal growth factor receptor promoter (24). The results presented in Fig. 4 suggest a similar mechanism of transcriptional repression by PML. In this case, PML represses transcription of the A20 gene by interfering with NFκB binding to the promoter. It is not clear why and how PML is involved in these two opposite functions in transcriptional regulation. Recent studies suggest that PML NB may serve as the storage site of important cellular regulatory proteins. Increased expression of PML may sequester these protein factors to the PML NBs and consequently limit their normal functional roles as transcription activators or repressors.

Although a role of PML in multiple pathways of apoptosis has been clearly established, the molecular mechanisms remain unclear. PML is required for Daxx-induced apoptosis in mouse splenocytes. In the absence of PML, cellular localization of Daxx was altered, and its proapoptotic function was impaired (36). In these cells, the ability of Daxx to sensitize Fas-induced apoptosis was abrogated (36). Daxx was also shown to specifically enhance Fas-induced apoptosis (38) in human cells by association with PML NB through a mechanism that involved the activation of caspase 8 and caspase 3; however, it is not known what role PML plays in TNF-induced apoptosis.

The results presented in this report support the novel mechanism of PML-induced apoptosis in inhibiting the expression of A20, a potent inhibitor of TNF-induced apoptosis. This study showed that PML represses the promoter activity of A20 induced by treatment with TNFα and PMA. PML inhibits transactivation of the A20 promoter by interfering with NFκB binding to its consensus cis-acting element. This finding was further supported by the study showing that stable overexpres-
A20 is also a potent inhibitor of NFκB, finally characterized as an inhibitor of TNF-induced apoptosis (25). It was originally characterized as an inhibitor of NFκB, repressing the expression of A20 and inhibiting its anti-apoptotic function. A20 is a novel zinc finger protein first described as an early responsive gene inducible by TNF treatment (25). It was originally characterized as an inhibitor of TNF-induced apoptosis (26). A20 is also a potent inhibitor of NFκB activation induced by not only TNF but also by interleukin-1, lipopolysaccharide, PMA, and hydrogen peroxide (39). This inhibitory effect may be mediated through its interaction with tumor necrosis factor receptor-associated factors (40) or other A20 binding proteins (41). Interestingly, the expression of A20 is controlled by NFκB, suggesting that it is involved in a negative feedback mechanism in regulating NFκB. NFκB is an important transcription factor that regulates the expression of many mediators of inflammation (42) and anti-apoptotic proteins involved in the survival pathway (43, 44). The finding that PML represses the transcription of the A20 promoter by interfering with NFκB binding is interesting. It will be important to further investigate the mechanism by which PML inhibits the binding of NFκB to its target DNA sequence and to examine whether the two proteins are functionally associated in vivo.

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Fig. 5. Overexpression of A20 inhibits TNFα/PML-induced apoptosis. A, inducible expression of A20 by Cd2+ in A20/U2OS stable cell line. A20/U2OS cells were treated with the indicated concentration of Cd2+ for 18 h, and total protein was isolated and subjected to Western blot analysis with anti-A20 and anti-tubulin antibodies. b and c, overexpression of A20 inhibits apoptosis induced by PML/TNFα. The inducible stable cell lines pMEP4/U2OS and A20/U2OS were treated (or untreated) with 5 μM Cd2+ and infected with Ad-C or Ad-PML (30). After 16 h, cells were treated (or untreated) with TNFα (20 ng/ml) and then allowed to continue culturing for another 8 h. Cell death was determined by trypan blue exclusion assay (b) and by caspase-7 (c) activation assay.
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