The promyelocytic leukemia protein PML regulates c-Jun function in response to DNA damage

Paolo Salomoni, Rosa Bernardi, Stephan Bergmann, Austin Changou, Sara Tuttle, and Pier Paolo Pandolfi

The promyelocytic leukemia (PML) gene, a tumor suppressor inactivated in acute promyelocytic leukemia (APL), regulates apoptosis induced by DNA damage. However, the molecular mechanisms by which PML modulates apoptosis following genotoxic stress are only partially elucidated. PML is essential for p53-dependent induction of programmed cell death upon γ-irradiation through PML-nuclear body (NB)–mediated control of p53 acetylation. Here, we show that PML selectively regulates proapoptotic transcription factors upon different types of DNA damage. We find that Pml inactivation protects fibroblasts from UV-induced apoptosis in a p53-independent manner. We demonstrate that c-Jun is required for UV-induced apoptosis and that PML is essential for both c-Jun transcriptional activation and DNA binding upon UV radiation. We find that PML physically interacts with c-Jun and that upon UV radiation the PML-NBs reorganize into novel nuclear microspeckled structures (UV-NBs), where PML and c-Jun dynamically accumulate. These data identify a novel PML-dependent pathway for c-Jun transcriptional activation and induction of apoptosis in response to DNA damage and shed new light on the role of PML in tumor suppression.

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Materials and methods

Apoptosis analysis

Mouse embryonic fibroblasts (MEFs) were fixed in acetone-methanol (1:4) and stained with propidium iodide (10 µg/mL). Subdiploid peak analysis was performed to evaluate the percentage of apoptotic cells. Alternatively, cell death was evaluated by trypan blue uptake. Mitochondrial transmembrane potential (∆Ψm) was measured by JC-1 staining following manufacturer’s instruction (Molecular Probes, Eugene, OR).

Western blotting and immunoprecipitation

MEFs were lysed in buffer A (50 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.6, 200 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 10 mM MgCl2, 10 mM MnCl2, 1% Triton-X100, 50 mM NaF, 0.5 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL leupeptin, aprotinin, and pepstatin). Antibodies used were anti-c-Jun-Ser 63 or -Ser 73 (Cell Signaling Technology, Beverly, MA), anti-c-Jun (BD Transduction Laboratories, San Diego, CA), anti-actin (Sigma, St Louis, MO), anti-HSP90 (BD Transduction Laboratories, San Diego, CA), p53 (Oncogene Science, Cambridge, MA), p53 Ser 18 (Cell Signaling Technology), p21 (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (Santa Cruz Biotechnology, Santa Cruz, CA), and p21 (Santa Cruz Biotechnology, Santa Cruz, CA).

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PML is required for UV-induced apoptosis

Since PML has been involved in several apoptotic pathways elicited by different cellular stresses, we set to determine whether PML also regulates apoptosis induced by UV irradiation. To this end, we exposed Pml<sup>+/+</sup> and Pml<sup>−/−</sup> MEFs to 60 J/m<sup>2</sup> UV light and found that the percentage of sub-G<sub>1</sub> hypodiploid cells was markedly impaired in Pml<sup>−/−</sup> MEFs (Figure 1A) and was dependent on neosynthesis (not shown). Reduction of apoptosis in Pml<sup>−/−</sup> cells was accompanied by a decrease in mitochondrial membrane potential depolarization (not shown). But contrast, similarly to previous reports, <sup>16,17</sup> p53<sup>−/−</sup> cells were not protected from UV-induced apoptosis at 60 J/m<sup>2</sup> (not shown), but they were more susceptible to UV-induced cell death at lower doses (not shown). Since p53 induction and activation by UV radiation trigger cell growth arrest through the up-regulation of p21,<sup>21</sup> we analyzed the UV-induced cell cycle arrest in Pml<sup>−/−</sup> cells. Pml<sup>−/−</sup> MEFs properly underwent cell cycle arrest (24 hours after 20 J/m<sup>2</sup>; not shown). Furthermore, Pml inhibition did not affect p53 induction or p53 Ser 18 phosphorylation (Figure 1B). p53 Target genes such as p21 and Bax were also normally induced at both mRNA and protein levels in Pml<sup>−/−</sup> cells (Figure 1B-C). Taken together these data demonstrate that PML regulates apoptosis upon UV radiation in a p53-independent manner.

Apoptosis triggered by cisplatin (cis-p), a chemotherapeutic agent that, similarly to UV radiation, induces the formation of DNA cross-links and can cause cell death in a p53-independent manner,<sup>23</sup> was also impaired in Pml<sup>−/−</sup> MEFs at all doses tested, although at a milder extent (Supplemental Figure S1, available at the Blood website; see the Supplemental Figures link at the top of the online article). These results indicate that PML can also regulate p53-independent apoptotic pathways induced by both UV radiation and cis-p.

Functional cross-talk between PML and c-Jun upon UV radiation

The c-Jun/INK-1 pathway has been involved in the regulation of UV-induced apoptosis.<sup>18,19,21</sup> To better clarify the role of c-Jun in UV-induced cell death and to test whether PML and c-jun would cooperate in this function, we, at first, used a dominant-negative mutant of c-Jun (DN–c-Jun), which inhibits c-Jun transcriptional activity.<sup>24</sup> Fibroblasts infected with a DN–c-Jun retroviral vector displayed reduction in cell death upon UV radiation (Figure 2A).

Results

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Importantly, the dominant-negative effect of DN–c-Jun on UV-induced cell death was significantly reduced in Pml+/−/− cells, suggesting a possible functional cooperation between PML and c-Jun (Figure 2A). Thus, UV-induced apoptosis requires intact PML and c-Jun functions. In this respect, it is worth noting that cis-platinum–induced apoptosis also depends on functional c-Jun.25

Because DN–c-Jun can in principle inhibit the transcriptional activity of other c-Jun family members, we analyzed whether c-Jun itself could potentiate UV-induced cell death. In unirradiated cells, c-Jun overexpression caused increased proliferation and reduced basal cell death in both wild-type and Pml+/−/− MEFs (not shown). By contrast, UV-induced cell death was further increased by c-Jun in wild-type MEFs, while c-Jun was ineffective in Pml+/−/− cells (Figure 2B). Taken together, these data demonstrate that c-Jun exerts a proapoptotic function upon UV radiation and that PML plays an important role in executing this function.

c-Jun transcriptional activity is impaired by Pml inactivation

We then studied the molecular mechanisms by which PML regulates this pathway. c-Jun transcriptional activity is enhanced following UV exposure.25 Therefore, we tested the effect of PML overexpression on c-Jun transcriptional activity in MEFs upon UV radiation. Upon UV irradiation, transactivation by a GAL4–c-Jun construct was induced in MEFs (Figure 3A). Overexpression of PML strongly potentiated UV-triggered c-Jun transcriptional activation (Figure 3A) in a dose-dependent manner (not shown). Importantly, coactivation of c-Jun by PML was only observed in irradiated cells, thus demonstrating that PML-mediated regulation of c-Jun transcriptional activity is UV radiation dependent (Figure 3A). We next compared GAL4–c-Jun activity in Pml+/+/+ and Pml+/−/− MEFs and found that the UV-dependent transcriptional activation of c-Jun was significantly impaired in the absence of PML (Figure 3B). This defect was directly due to the absence of PML, since reintroduction of PML into Pml+/−/− MEFs almost completely rescued c-Jun–regulated transcription (Figure 3B) in a dose-dependent fashion (not shown). We next studied the transcriptional activity of endogenous c-Jun upon UV radiation. To this end, we tested 2 types of c-Jun responsive elements: multimerized TPA-responsive elements and CRE-like sequences, which bind c-Jun/ATF-2 dimers.26,27 TRE activity was not significantly increased in untreated cells (Figure 3D). By contrast, CRE basal activity was clearly enhanced in Pml+/+/+ MEFs upon UV radiation, while, once again, in Pml+/−/− MEFs its transactivation was completely impaired (Figure 3C).

We next investigated whether PML influences c-Jun transcriptional activity by regulating its DNA binding ability and found that, upon UV irradiation, the c-Jun/ATF-2 binding activity was clearly induced in wild-type MEFs, while, remarkably, it was impaired in Pml+/−/− cells (Figure S2). Supershift analysis using anti–c-Jun and anti–ATF-2 antibodies confirmed the presence of both c-Jun and ATF-2 in the CRE complex (Figure S2). Similar results were obtained in UV radiation cross-linking experiments (not shown).

We next set to determine whether c-Jun DNA binding activity was influenced by Pml inactivation in vivo by chromatin immunoprecipitation experiments (ChIP) using an anti–c-Jun antibody. We studied the binding of c-Jun to its own promoter, which contains 2 CRE sequences, termed jun1 and jun2.27 The binding of c-Jun to both jun1 and jun2 promoter regions was induced by UV radiation in Pml+/+/+ cells (Figure 3D). By contrast, we observed a reproducible and consistent reduction of c-Jun binding to both the jun1 and jun2 in Pml+/−/− cells (Figure 3D).
Figure 4. PML relocates into novel microspeckles upon UV radiation. (A) MEFs were UV-irradiated (60 J/m²) and then stained with an antibody against mouse PML. Nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole). (B) PML is delocalized upon cisplatin treatment. WI-38 cells were treated with cis-pl at 10 μg/mL for 6 hours and subsequently fixed and stained with an antibody against human PML (green). Nuclei were visualized by DAPI. (C–E) Analysis of PML-NB components in UV-irradiated fibroblasts. (C) WI-38 cells grown on coverslip were UV-irradiated (60 J/m²), stained with antibodies anti-PML (green) and anti-SUMO1 (red), and analyzed by confocal fluorescence microscopy. Nuclei were visualized by DAPI (blue). Colocalization of PML and SUMO1 is shown (yellow). (D) WI-38 cells were stained with antibodies against PML (green) and DAXX (red). Colocalization of PML and DAXX is shown (yellow). (E) Cells were stained with antibodies against PML (green) and CBP (red). Colocalization of PML and CBP is shown (yellow).

PML localization is disrupted upon UV irradiation

We then analyzed the effects of UV irradiation on the PML-NB. In unirradiated MEFs, PML was found typically concentrated in 10 to 15 PML-NBs/nucleus (Figure 4A). Strikingly, PML relocalized into multiple microspeckles upon UV radiation in both MEFs and primary human fibroblasts (Figure 4A, and not shown). This phenomenon affected more than 60% of cells. By contrast, treatment of MEFs with γ-rays did not result in such changes (data not shown). A recent study reported that PML relocation upon UV exposure is p53 dependent in tumor cell lines. Nevertheless, PML is able to relocalize to microspeckles in p53−/− MEFs, thus indicating that this phenomenon is not p53 dependent in primary cells (not shown). Similar to UV radiation, cis-pl treatment resulted in PML delocalization (Figure 4B). The UV-induced microspeckles partitioned with the nuclear insoluble fraction and the effect was not accompanied by an increase in PML protein levels (not shown). We next studied the localization of other PML-NB components. SUMO1 and DAXX were found to relocate along with PML into the UV-induced microspeckles (Figure 4C–D). While CBP and p53 accumulate into the PML-NB upon γ-rays, they were not found to accumulate in the UV-induced microspeckles (Figure 4E, and not shown). Thus, the regulation of c-Jun transcriptional function by PML occurs along with its delocalization from the PML-NB into novel microspeckled nuclear structures, hereafter referred to as UV-nuclear bodies (UV-NBs). A recent report demonstrated that PML deSUMOylation results in the reorganization of the PML-NB and in c-Jun activation. However, we were unable to detect changes in PML SUMOylation upon UV radiation (not shown). Moreover, SUMO1 localizes to the UV-NB (Figure 4C), suggesting that PML delocalization upon UV radiation might be triggered by different signals or posttranslational modifications. Overall, this evidence suggests that the function of PML upon UV radiation is PML-NB independent.

PML and UV-activated c-Jun colocalize and physically interact

As PML regulates p53 function in the PML-NBs upon γ-ray irradiation, we next set to determine whether UV light would cause c-Jun to colocalize with PML in the UV-NBs. While an antibody against total c-Jun showed a rather diffuse staining upon UV radiation (not shown), an antibody recognizing phosphorylated c-Jun (P–c-Jun) detected nuclear microspeckles in more than 80% of irradiated cells, in both primary human fibroblasts and MEFs (Figure 5A, and not shown). Remarkably, a statistically significant colocalization of P–c-Jun and PML in the UV-NB was found in primary fibroblasts at the endogenous level (Figure 5A; Figure S3). These nuclear speckles might potentially represent sites of active c-Jun–dependent transcription. In agreement with the fact that UV induces c-Jun phosphorylation, we did not observe P–c-Jun staining in the majority of unirradiated cells, and P–c-Jun was never found in the PML-NB in untreated cells (not shown). Since c-Jun and PML colocalize upon UV radiation, we tested whether they physically interact in vivo and observed that endogenous PML and c-Jun proteins were readily communoprecipitated from UV-irradiated fibroblasts (Figure 5B). To test whether the c-Jun/PML interaction was direct, we performed GST pull-down experiments by using GST–c-Jun and in vitro–translated PML. GST–c-Jun and PML directly interacted (Figure 5C). We next identified the RBCC N-terminal domain as the
PML region responsible for the PML-c-Jun interaction, since a PML mutant lacking the RBCC did not interact with GST-c-Jun (Figure 5C).

Discussion

Accumulating evidence indicates that PML exerts its tumor suppressive function by regulating cellular senescence and promoting apoptosis. PML is part of a p53-controlled tumor suppressive pathway for the induction of senescence upon oncogenic transformation. Furthermore, PML and p53 functionally interact during apoptosis induced by γ-irradiation in thymocytes. However, PML also controls apoptotic pathways that do not rely on p53. The mechanisms regulating the induction of apoptosis upon UV irradiation has been the object of intense research efforts in the past 2 decades. Several cellular pathways can modulate this process. The p53 tumor suppressor has been shown to mainly modulate cell cycle arrest in UV-irradiated primary cells, while its role in apoptosis is unclear. Disruption of the c-Jun/JNK pathway results in alteration of apoptosis upon UV radiation. However, although the c-Jun/JNK pathway has been implicated in the regulation of apoptosis upon UV radiation, the precise molecular mechanisms underlying its activation upon cellular stress remain largely unknown. In the present report, we proved that c-Jun indeed mediates UV-triggered cell death and provide direct evidence that PML selectively regulates distinct transcription factors upon different DNA-damaging agents: p53 upon γ-irradiation and c-Jun upon UV irradiation (Figure 5D). Importantly, we demonstrate that PML modulates the proapoptotic function of c-Jun upon UV radiation by potentiating transcriptional activity.

Strikingly, UV light induces the disruption of the UV-NB and the formation of novel microspeckled structures, the UV-NB, in which PML and phosphorylated c-Jun dynamically accumulate. The formation of these structures does not rely on p53, since it normally occurs in UV-irradiated p53−/− MEFs (not shown). By contrast, upon γ-rays the PML-NB is not disrupted and serves a critical role as a center for p53 modification and transcriptional activation. On the basis of our data, we, therefore, propose a model by which the nuclear dynamics of PML upon distinct apoptotic stimuli dictate the selective activation of different proapoptotic transcription factors.

Interestingly, while the PML tumor suppressor regulates c-Jun activity in a strict UV-dependent fashion, we find that the PML-RARα oncoprotein of APL acts as a constitutive and UV-independent c-Jun transcriptional coactivator (data not shown). Thus, it could be envisioned that PML-RARα constitutively triggers the oncogenic potential of c-Jun, while PML would specifically regulate c-Jun proapoptotic function modulating its UV-dependent role. As point mutations of the PML gene have been recently discovered in aggressive cases of APL, it would be intriguing to test whether these mutants are defective in activating c-Jun upon DNA damage, thus further protecting APL cells from cell death induced upon DNA damage. Furthermore, this pathway could be altered in solid tumors, as cancers of various histologic origins have been found to lack expression of the PML protein.

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References

17. Lackinger D, Kaina B. Primary mouse fibroblasts deficient for c-Fos, p53 or for both proteins are hypersensitive to UV light and alkylating agent-induced chromosomal breakage and apoptosis. Mut Res. 2000;457:113-123.
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