The Promyelocytic Leukemia Protein Protects p53 from Mdm2-mediated Inhibition and Degradation*

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The p53 protein is kept labile under normal conditions. This regulation is governed largely by its major negative regulator, Mdm2. In response to stress however, p53 accumulates and becomes activated. For this to occur, the inhibitory effects of Mdm2 have to be neutralized. Here we investigated the role of the promyelocytic leukemia protein (PML) in the activation of p53 in response to stress. We found that PML is critical for the accumulation of p53 in response to DNA damage under physiological conditions. PML protects p53 from Mdm2-mediated ubiquitination and degradation, and from inhibition of apoptosis. PML neutralizes the inhibitory effects of Mdm2 by prolonging the stress-induced phosphorylation of p53 on serine 20, a site of the checkpoint kinase 2 (Chk2). PML recruits Chk2 and p53 into the PML nuclear bodies and enhances p53/Chk2 interaction. Our results provide a novel mechanistic explanation for the cooperation between PML and p53 in response to DNA damage.

The p53 tumor suppressor is important for the control of cell growth arrest, senescence, and cell death. These functions of p53 are pivotal for cellular response to stress signals, such as DNA damage (reviewed in Refs. 1 and 2). During tumorigenesis these cellular responses are abrogated by a loss of p53 function, either directly by mutations or indirectly by modifications of p53 inhibitors, such as Mdm2 (Hdm2 in human). The p53 protein is tightly regulated. Under normal conditions it is kept labile, but upon exposure to stress, p53 is transiently accumulated in an active form within the nucleus. This rapid activation occurs at multiple levels including protein stabilization, nuclear accumulation, and post-translational modifications. The regulation of p53 involves several auto-regulatory loops in which Mdm2 performs a pivotal role. In the p53-Mdm2 feedback loop, activated p53 induces mdm2 transcription, which in turn binds p53, blocks its functions, and promotes p53 degradation via the ubiquitin-proteasome pathway. It is imperative that p53 be relieved from inhibition by Mdm2 to be efficiently and properly activated in response to stress. Several mechanisms have been shown to interrupt this loop, including post-translational modifications of p53 and Mdm2, subcellular transportation, and interaction with specific modulators (reviewed in Ref. 4).

The inhibitory influence of Mdm2 on p53 is affected by phosphorylation of both proteins. Phosphorylation of Mdm2 by Akt enhances Mdm2-mediated inhibition of p53 (reviewed in Ref. 3), whereas phosphorylation by ATM1 (5) or c-Abl (6) impairs this inhibition. Likewise, the phosphorylation of p53 on Ser-20, which resides within the Mdm2-binding site, reduces the binding affinity between Mdm2 and p53, hence leading to the accumulation and activation of p53 (7, 8). This phosphorylation is mediated by checkpoint kinase (Chk) 2 in response to ionizing radiation (IR) and by Chk1 or JNK in response to UV light (7–9). Thus, specific phosphorylation of p53 and Mdm2 modulates their interplay.

The subcellular distribution of p53 and Mdm2 is also critical for their regulation (reviewed in Ref. 10). The nuclear/cytoplasmic translocation of p53 provides a broad level of control; however, shuttling within the nucleus achieves the fine-tuning in p53 activation. This nuclear transportation is mediated by PML, a protein that is fused to the retinoic acid receptor α in patients with acute promyelocytic leukemia (APL) (11). PML is essential for the formation of subnuclear structures called PML nuclear bodies (PML-NBs), which contain a variety of proteins including Daxx, pRb, and p53. The latter is recruited into the PML-NBs in response to Ras activation, IR, or UV light and As2O3 (12–16); Fig. 5 in this study). PML is critical for the proper activation of p53 transcriptional activity and for its apoptotic activity (12–16). The underlying mechanism for this activation has attracted much interest. To this end, several p53 regulators have been found to be associated with the PML-NBs. The CREB-binding protein (CBP) is recruited into the PML-NBs where it acetylates p53 at lysine 382 (Lys-382), thereby enhancing its transcriptional activity (16). This acetylation is facilitated by the prior phosphorylation of p53 on Ser-46 by the human serine/threonine kinase homedomain-interacting protein kinase-2 (HIPK2), which is also localized to the PML-NBs (17, 18). More recently, these modifications were also proposed to contribute to p53 stabilization (19). However, the substitution of Lys-382 to arginine reduced, but did not abolish, the induction of p53 transcriptional activation by PML (16). Further, Ser-46 is not conserved between human to mouse p53.
and Ser-46 phosphorylation is not triggered in response to IR (18). Thus, although these modifications are important for the regulation of p53, they may not be sufficient to explain the severe impairment of p53 transcriptional activity in the PML null mice and the high resistance of these mice to IR-induced apoptosis (15, 20). It is likely, therefore, that an additional mechanism(s) exists to explain this cooperation.

Because Mdm2 is the major player in p53 regulation, and PML is a positive regulator of p53, we investigated the possibility that PML may activate p53 by antagonizing the inhibitory effects of Mdm2. Here we demonstrate that PML is essential for the accumulation of p53 in response to DNA damage. This action of PML involves the neutralization of Mdm2-dependent ubiquitination and degradation of p53 and the inhibition of p53-mediated apoptosis. PML impairs Mdm2 effects, at least in part, by prolonging the phosphorylation of p53 on Ser-20. This process is facilitated by the recruitment of Chk2 into the PML-NBs. Overall our results demonstrate a new mechanistic explanation for the activation of p53 by PML in response to DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Assays—H1299 lung adenocarcinoma cells, Saos2 osteosarcoma cells, and NB4 acute promyelocytic leukemia cells (21) were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum. Primary embryo fibroblasts from normal mice and from PML knock-out mice were prepared and grown as described previously (20). Mouse embryo fibroblasts (MEFs), 293 kidney epithelial cells, and 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal calf serum.

AsO$_3$ was kept as 1 mM stock solution in phosphate-buffered saline. Cells were incubated with 0.1–1 mM As$_2$O$_3$ (4 h for NB4), and where indicated, cells were also exposed to IR (5 Gy). DNA damage was induced by incubation of cells with mitomycin C (5 µg/ml, Sigma) or IR (5–10 Gy). Transfections by the calcium phosphate precipitation method and retroviral infections were carried out as described previously (22). The same amount of plasmid DNA was kept in each sample by supplementing with empty plasmid.

**Plasmids**—Expression plasmids used in this study were: human wt p53 (pCMV-Neo-BamHI-p53) and human wt p53-GFP (pEGFP-N1 fused to p53 (23), mutant p53Ala-20 (pRC/CMV Alav-20) (24), mouse wt Mdm2 (pCOC-Mdm2 X2) (22), Hdm2 (pCMV-Neo-BamHI HDM2), human wt PML-IV (formerly pCDNA3PML3), wt FLAG-PML-IV (pSGS PML), FLAG-PML-RARα, FLAG-PML-RBCC (15), wt GFP-PML-IV (pEGFP-N1 fused to PML-IV), pBabe-puro wt GFP-PML, pBabe-puro GFP, wt FLAG-PML-R2, and wt FLAG-PML-R3 (15), pBabe-puro GFP, wt FLAG-Chk2 (pCDNA3 Chk2), and pEGFP-N1 alone.

**Immunoprecipitation Assay and Western Blot Analysis**—Western blot analysis was carried out as described previously (22) with the exception that for the detection of Ser-20 phosphorylation, cells were harvested and lysed directly in protein sample buffer followed by sonication. Immunoprecipitation assay was carried out essentially as described previously (6).

For the in vivo ubiquitination assay, H1299 cells were transfected with a combination of expression plasmids for p53 (1 µg), Mdm2 (1 µg), and PML-IV (3 µg). Twenty-four hours after transfection, cells were treated with the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN, 150 µM, Calbiochem) for 4 h and with As$_2$O$_3$ (1 µM, Sigma) for 4 h where indicated. Cells were harvested and cell extracts resolved by SDS-PAGE followed by blotting with anti-p53 antibodies (DO1 and PAB1801). The p53 ubiquitin conjugates were detected by the smear and high molecular weight bands of p53 as described previously (26).

The antibodies used in this study were: anti-human p53 monoclonal antibodies PAb1801 and DO1, anti-mouse polyclonal antibodies CM5 (NovoCastra), anti-PML monoclonal antibody PG-M3 (DAKO), anti-phospho-p53 Ser-20 polyclonal antibody (Cell Signaling), anti-FLAG monoclonal antibody (Sigma), anti-Chk2 (DCS-273, Sigma; H-300, Santa Cruz); anti-actin (Ac-40 Sigma), anti-Bax (Ab-5, Oncogene Research Products), anti-α-tubulin DM1A (Sigma), horseradish peroxidase-labeled goat-anti-mouse IgG (Jackson Immunoresearch Laboratories), anti-GFP (Roche Applied Science), and Envision peroxidase anti-mouse or anti-rabbit IgG anti-mouse (Dako Corp.).

**Immunofluorescent Staining Analysis**—For immunofluorescent staining, H1299 and Saos-2 cells were plated on glass coverslips. Twen-ty-four hours post-transfection, cells were fixed in cold methanol and stained with anti-FLAG or anti-PML (P93M) followed by Cy5-conju- gated goat anti-mouse secondary antibody. GFP-p53 and GFP-PML-IV were detected by GFP fluorescence. DNA was stained with DAPI (4’,6’-diamidino-2-phenylindole). The stained cells were observed with a fluorescein microscope (Nikon).

**Apopotic Assay**—The apoptotic assay was carried out essentially as described previously (24). Samples were analyzed by a cell sorter (FACSCalibur) using CellQuest software (BD Biosciences).

**RESULTS**

**PML Is Critical for p53 Accumulation in Response to DNA Damage**—p53 protein is accumulated in response to DNA damage or constitutive Ras activation (1). These stress conditions also trigger the activation of p53 by PML (13–16). It was therefore tempting to suggest that PML activates p53 by enhancing its accumulation. To address this question under physiological conditions, the accumulation of endogenous p53 in response to DNA damage was measured. Primary mouse embryo fibroblasts derived from normal mice (PML+) or from PML null mice (PML−) were exposed to mitomycin C (3 µg/ml). At different times (3–12 h) after exposure the steady state levels of p53 was measured by Western blot analysis using anti-p53 polyclonal antibodies (CM5). In normal cells, p53 levels were elevated by 7 h and continued to accumulate at 12 h after treatment (Fig. 1A, lanes 1, 5, and 7). By contrast, the accumulation of p53 in PML null cells was dramatically impaired even after 12 h (Fig. 1A, lanes 2, 6, and 8). Similar results were obtained when cells were exposed to a different DNA damage agent, IR (5 Gy). In normal MEFs, the accumulation of p53 was seen clearly by 1 h after irradiation and was further increased by 2 h (Fig. 1B, lanes 1–5). On the other hand in PML-deficient MEFs the accumulation of p53 was severely impaired (Fig. 1B, lanes 8 and 9). The maximal levels of p53 in these cells were significantly lower than in normal cells (Fig. 1B).

To strengthen this finding, the PML-deficient MEFs were reconstituted with PML. For this purpose, cells were infected with retrovirus expressing either GFP-PML or GFP alone. Forty-eight hours after infection cells were exposed to IR (5 Gy), and p53 protein levels were monitored. Consistent with the results in Fig. 1B, efficient accumulation of p53 in response to IR was seen in the PML-reconstituted cells but not in the GFP-expressing control cells (Fig. 1C). Further, we examined the transcriptional activity of p53 in these cells in response to IR. To this end, the induction of the p53 target gene, Bax, was compared between MEFs lacking PML and the PML-reconstituted cells. The induction of Bax was measured in the cell extract from the experiment described in Fig. 1C. Elevation of the Bax protein was seen in response to IR only in PML-reconstituted cells (Fig. 1D, lanes 5–8) but not in the control cells (lanes 1–4). Taken together, these results clearly demonstrate a critical role for PML in the rapid and efficient accumulation of active p53 in response to DNA damage.

**PML Protects p53 from Inhibition by Hdm2**—How does PML promote p53 accumulation in response to DNA damage? Because Hdm2 is the major determinant in the degradation of p53 (27, 28), we asked whether PML activates p53 and enhances its accumulation by antagonizing the inhibitory effects of Hdm2 on p53. This notion was addressed at the levels of p53 protein stability and p53 activity.

**PML Impairs Hdm2-dependent p53 Degradation**—To test whether PML protects p53 from Hdm2-mediated degradation, H1299 cells were transfected with low amounts of p53 expression plasmid alone (Fig. 2A, lane 1), together with Hdm2 (lanes 2–5), or together with Hdm2 and increasing amounts of PML-IV (lanes 3–5). The PML-IV isoform (according to the new nomenclature for PML (29)) was used throughout this study.
 Twenty-four hours post-transfection, cells were harvested, and the expression level of the p53 protein was determined by Western blot analysis. Co-expression of Hdm2 markedly reduced the expression level of p53 (Fig. 2A, lane 2); however, the addition of PML-IV protected p53, at least partially (lanes 3–5). Thus, co-expression of PML-IV protects p53 from the rapid and efficient degradation by Hdm2.

Because PML interacts with p53 (14, 15), the requirement for this interaction for the protection of p53 from Hdm2 was determined. To this end, two PML mutants, PML-RAR and PML-RBCC, which are deficient in p53 binding (15), were compared with wt PML for their ability to protect p53 from Hdm2-mediated degradation. The degradation assay described in Fig. 2A was used for this purpose. Although wt PML protected p53 from degradation by Hdm2, neither PML-RAR nor PML-RBCC was able to protect p53 from degradation (Fig. 2B). This result suggests that interaction between p53 and PML is essential for its protection from Hdm2.

Mdm2 promotes p53 degradation through the ubiquitin proteasome pathway (27, 28). The ubiquitin-protein isopeptide ligase (E3) of Mdm2, mediated by the RING-finger domain, is crucial for this function (30). The partial protection of p53 from Hdm2-mediated degradation raised the question whether PML-IV interferes with the ubiquitination of p53 by Hdm2. To address this question in vivo, H1299 cells were transfected...
with p53, p53 and Hdm2, or both together with PML-IV. Twenty-four hours after transfection, cells were treated with ALLN for 4 h to prevent p53 degradation. To measure the effect of endogenous PML on this activity of Hdm2, cells were treated with arsenic trioxide (As$_2$O$_3$) for 4 h, which activates PML by promoting its SUMO (small ubiquitin modifier) modification (31). Following these treatments, cells were harvested and p53 ubiquitin conjugates were detected by Western blot analysis using anti-p53 antibodies. Co-transfection of Hdm2 together with p53 enhanced the ubiquitination of p53 as judged by the high molecular weight bands and smear (Fig. 2C, lanes 1 and 2). The treatment of cells with As$_2$O$_3$ impaired the in vivo ubiquitination of p53 by Hdm2 (lane 3). The addition of exogenous PML-IV strongly reduced the extent of p53 ubiquitination (Fig. 2C, lane 4) and, when combined with As$_2$O$_3$ treatment (lane 5), totally blocked this effect of Hdm2. These results show that PML protects p53 by preventing its in vivo ubiquitination by Hdm2.

PML Relieves the Inhibitory Effect of Hdm2 on p53-dependent Apoptosis—Our finding that PML-IIV protects p53 from Hdm2-mediated degradation prompted us to evaluate the biological consequences of this protection. Because PML plays an important role in p53-dependent apoptosis (15) and Hdm2 inhibits this activity of p53 (22, 32), we chose to examine whether PML relieves p53-dependent apoptosis from inhibition by Hdm2. For this purpose we employed a transient transfection apoptotic assay for p53 (22). Initially, the cooperation between p53 and PML-IV in the induction of apoptosis was measured in H1299 cells transfected with p53 or PML-IV alone or with both together. Sixty hours post-transfection, cells were harvested, stained for p53 or PML-IV, and subjected to flow cytometric analysis. Apoptosis induced by the transfection conditions or by GFP was determined by transfecting H1299 cells with expression plasmid for GFP alone. The GFP-positive subpopulation exhibited an average of 3 ± 1.2% apoptosis (data not shown). Expression of wt p53 induced 10% apoptosis (Fig. 3A, I), whereas PML-IV induced only 3% (Fig. 3A, II). However, co-expression of p53 and PML-IV induced 26% apoptosis, indicating a synergistic effect (Fig. 3A, III and IV). These results are in accordance with the role of PML-IV in p53-dependent apoptosis (15).

The effect of PML-IV on the inhibitory effect of Hdm2 on p53-dependent apoptosis was next determined by introducing Hdm2 into this apoptotic assay. In this case, H1299 cells were transfected with larger amounts of p53 expression plasmid (3 μg) to induce at least 20% apoptosis (Fig. 3B, I). The addition of Hdm2 expression plasmid (5 μg) reduced the level of apoptosis by 50% (Fig. 3B, II), but this inhibition was relieved, to a large extent, by co-transfection with PML-IV expression plasmid (3 μg). These results (summarized in Fig. 3B, IV) demonstrate that PML-IV protects p53 apoptotic activity from the inhibitory effect of Hdm2. Taken together these data support the notion that the accumulation and activation of p53 by PML-IV involves the neutralization of Hdm2.

PML Promotes the Phosphorylation of p53 on Ser-20—Next we searched for a mechanism to explain the neutralization of Hdm2 by PML. We and others have previously shown that phosphorylation of p53 on Ser-20 plays a critical role in the modulation of the p53-Hdm2 feedback loop (reviewed in Ref. 33). It was tempting to suggest that PML protects p53 from Hdm2 by facilitating this phosphorylation. To test this notion we employed different experimental systems. First, H1299 cells lacking p53 expression were transfected with expression plasmids for p53 alone or together with increasing amounts of FLAG-tagged PML expression plasmid (1 and 3 μg). Twenty-four hours after transfection cells were exposed to IR (10 Gy), and 30 min later they were harvested for Western blot analysis. As a control, cells transfected with p53 alone were kept untreated. The extent of p53 phosphorylation on Ser-20 was determined by Western blot analysis using anti-p53 phospho-Ser-20 antibody. The transfection of p53 alone induced significant levels of phosphorylation, presumably because of the effective DNA damage signal generated by the transient transfection of DNA (34, 35) (Fig. 4A, lane 1). This relatively high level of phosphorylation was not increased further after exposure to IR, at least not at this time point. However, the addition of PML increased the level of phosphorylation (by 2-fold as measured by densitometry; Fig. 4A, lanes 3 and 4). Higher levels of PML did not cause a further increase, which apparently had reached a maximal level already at 1 μg of PML expression plasmid. This result supports a role for PML in enhancing Ser-20 phosphorylation of p53 in response to IR.

Second, we evaluated whether the observed effect of PML on Ser-20 phosphorylation holds at physiological levels of p53 and PML. For this purpose we used the APL-derived cell line, NB4, which contains the t(15;17) translocation expressing PML-
time after IR. The presence of As$_2$O$_3$ increased the level of Ser-20 phosphorylation at 1 and 1.5 h by 3- and 2-fold, respectively (Fig. 4B, lane 5 versus 6 and 7 versus 8). Therefore, reactivation of endogenous PML by As$_2$O$_3$ (31) prolonged the IR-induced phosphorylation of p53 on Ser-20 in NB4 cells. In these cells p53 is mutated (36), and hence it does not induce the expression of target genes including Mdm2. Consequently, the expression of p53 remains unchanged upon exposure of NB4 cells to IR. This facilitates the measurement of p53 phosphorylation without the need to compensate for IR-induced changes in the levels of p53. It should be noted that treatment of NB4 with either 10-fold higher levels of As$_2$O$_3$ (1 µM) or with higher dose of IR (10 Gy) extends the duration of Ser-20 phosphorylation (data not shown). The third experimental system used to measure the effect of PML on the extent of p53 Ser-20 phosphorylation was based on MEFs lacking PML (infected with GFP only) and those reconstituted with PML (GFP-PML). The two types of cells were exposed to IR (10 Gy), and phosphorylation at Ser-20 was determined as described above. As shown in Fig. 4C, efficient phosphorylation of p53 on Ser-20 was observed in the PML reconstituted cells (lanes 4–6) but not in MEFs lacking PML (lanes 1–3).

A different approach was used to examine more directly the link between PML and p53 Ser-20 phosphorylation as a mechanism for evading Hdm2. For this purpose the protection of p53 from Hdm2-mediated p53 degradation by PML was compared between wt p53 and the substitution mutant p53Ala-20, which cannot be phosphorylated on Ser-20 (24). H1299 cells were transfected with expression vectors for wt p53 or p53Ala-20 alone, or together with Hdm2 alone, or Hdm2 and PML-IV. Twenty-four hours post-transfection, cells were harvested and the levels of p53 were monitored by Western blot analysis using anti-p53 antibodies (DO1 and PAh1801). The degradation of p53 by Hdm2 was partially prevented by PML-IV (Fig. 4D, lanes 1–3). In contrast, PML-IV failed to protect p53Ala-20 from degradation by Hdm2 at any measurable level (Fig. 4D, lanes 4–6). This dramatic difference between the effects of PML-IV on wt p53 versus p53Ala-20 was observed despite the higher levels of PML-IV expression in the p53Ala-20 sample than in the p53 wt sample (Fig. 4D, lanes 3 versus 6). These results lend further support for the role of PML-IV in the phosphorylation of p53 on Ser-20 and provide compelling evidence for a critical role of Ser-20 in the activation of p53 by PML-IV and its protection from Hdm2.

**Chk2 Interacts with PML and Is Recruited into the PML-NBs**—The phosphorylation of p53 on Ser-20 in response to IR is mediated by Chk2 (7, 8). The accumulation and activation of p53 in response to IR are impaired in Chk2-deficient cells (37) and in PML null cells (Fig. 1). Further, both Ser-20 phosphorylation (24) and PML (Figs. 2 and 3) impair the inhibitory effects of Mdm2 on p53, and PML prolongs Ser-20 phosphorylation in response to IR (Fig. 4). It is therefore tempting to propose that PML facilitates the interaction between p53 and Chk2. One possible mechanism of facilitation may involve the recruitment of both proteins into the PML-NBs in response to a stress signal. To explore this possibility, we initially examined the effect of PML on the redistribution of p53 and Chk2 in response to IR. Saos-2 cells were transfected with expression plasmids for GFP-p53 together with expression plasmid for PML. Twenty-four hours after transfection, cells were either untreated or exposed to IR (10 Gy) and 1 h later were fixed and subjected to immunofluorescent staining. p53 was detected by GFP fluorescence, and PML was stained with anti-PML (PGM3) followed by Cy5-conjugated secondary antibody. The diffused nuclear pattern of p53 in the untreated cells (Fig. 5A) formed a distinct speckled pattern after exposure to IR (Fig. 5B).
The recruitment of both Chk2 and p53 into the PML-NBs, together with the effect of PML on Ser-20 phosphorylation of p53, suggested that PML facilitates the interaction between Chk2 and p53. This possibility was examined in NB4 cells before and after PML activation. NB4 cells were either left untreated or exposed to As2O3 (1 μM) for 4 h. The Chk2 protein was immunoprecipitated from the cell extracts, and the amount of the p53 protein that was bound to the Chk2 immune complex was determined by Western blot analysis. Activation of PML in NB4 cells by As2O3 treatment increased the amount of p53 bound to Chk2 (Fig. 6, I). This increase was not due to changes in the expression levels of Chk2, either in the immune complex (Fig. 6, II) or in the cell extracts (IV), or to changes in the levels of p53 (III). These results suggest that the presence of active PML enhances the interaction between Chk2 and p53, presumably by recruiting both proteins into the PML-NBs.
In response to stress, p53 has to be relieved from Hdm2 in order to be activated in the proper time and place. In this study we evaluated the role of PML in the modulation of the p53-Hdm2 feedback loop and demonstrated an important role for PML in the accumulation of an active p53 protein in response to DNA damage (Fig. 1). The drastic impairment in the accumulation of p53 in PML-deficient primary fibroblasts explains the defective transcriptional and apoptotic activities of p53 under these conditions, as well as the resistance of PML null mice to IR (15, 20). Because Hdm2 is the major regulator of p53 stability (27, 28), we investigated whether PML governs p53 accumulation by antagonizing this action of Hdm2. We demonstrated that PML impairs the ability of Hdm2 to promote the in vivo ubiquitination of p53 (Fig. 2B). This is consistent with the protection of p53 from Hdm2-mediated degradation (Fig. 2) and inhibition of p53-dependent apoptosis (Fig. 3). Several mechanisms have been described to explain the release of p53 from Hdm2, including the phosphorylation of p53 (reviewed in Refs. 4 and 38). Among these phosphorylation sites, Ser-20 poses as an attractive candidate for modulation by PML because phosphorylation of this site reduces the binding affinity between p53 and Hdm2, leading to the accumulation and activation of p53 (33). We demonstrated that the presence of functional PML is important for the maintenance of p53 phosphorylation at Ser-20 in response to IR (Fig. 4) and that the protection of p53 by PML requires Ser-20 (Fig. 5). These findings help to explain how PML protects p53 from Hdm2.

Upon exposure of cells to IR, p53 is phosphorylated on Ser-20 by Chk2 (7, 8). The physiological relevance of this phosphorylation has been demonstrated by two lines of evidence. First, the accumulation of p53 and its activation is severely impaired in Chk2-deficient cells (37). This impairment is confined to the apoptotic activity and apparently not to the induction of G1 growth arrest by p53 (39). Second, human cancers bearing germ-line or somatic mutations in the Chk2 gene often lack p53 alterations, unlike the high frequency of p53 mutations in other types of cancers (reviewed in Ref. 33). The significance of these observations in human cells remains to be defined in mice where it is unclear whether the human Ser-20 equivalent (mouse Ser-23) performs the same regulatory functions. A substitution of the mouse equivalent, Ser-23, with Ala-23 in embryonic stem cells had no significant effect on the activation or accumulation of p53 in response to DNA damage (40). This apparent controversy may reflect differences in the regulatory role of Ser-20 in human p53 versus in mouse Ser-23. It also raises the likely possibility that phosphorylation of Ser-20 is not the only mechanism by which Chk2 activates p53 in response to DNA damage. Further studies are required to unravel this species-related difference.

We have shown here that PML plays an important role in promoting co-localization and physical interaction between p53 and Chk2. Exposure of cells to IR induced the transportation of both p53 and Chk2 into the PML-NBs where they co-localized with PML (Fig. 5). Further, Chk2 and p53 co-localized in the PML-NBs under these stress conditions. This shuttling to the PML-NBs coincides with the phosphorylation of p53 on Ser-20 by Chk2 in response to IR (Fig. 4), supporting a role for PML in facilitating this stress-induced phosphorylation of p53. The recruitment of p53 into the PML-NBs requires the binding of PML to p53 (14, 15). Likewise, this interaction is required for the protection of p53 from Hdm2 (Fig. 2), suggesting that the recruitment of p53 by PML to the NBs is essential for the protection of p53. The finding that PML also interacts with Chk2 (41) (data not shown) suggests that this interaction may also be required for the recruitment of Chk2 into the PML-NBs. Importantly activation of PML enhances the interaction between p53 and Chk2 (Fig. 6), thereby facilitating Ser-20 phosphorylation of p53 by Chk2. It was recently reported that Chk2 binds and phosphorylates PML on Ser-117, a phosphorylation that promotes the p53-independent apoptotic activity of PML (41). It was also shown that Chk2 co-localizes with PML in the PML-NBs irrespective of stress conditions. However, we find that the speckled distribution of Chk2 and its co-localization with PML is induced by exposure to IR (Fig. 5), consistent with previous studies showing a diffuse nuclear distribution of Chk2 in the absence of IR (42–44). This difference is yet to be resolved. Nevertheless, it appears that the interaction of Chk2 with PML and its recruitment to the PML-NBs contribute to the cellular response to stress in a p53-dependent (this study) pathway as well as in p53-independent pathways (41). While this manuscript was under revision, a paper by Wei et al. (45) showed an interaction between PML and Mdm2. Whether this interaction contributes to the protection of p53 from Mdm2 is yet to be demonstrated.

On the basis of our findings, we propose a model whereby cells exposed to IR, PML binds to and recruits p53 and Chk2 into the PML-NBs. This transportation facilitates the phosphorylation of p53 on Ser-20 by Chk2, thereby relieving p53 from inhibition by Hdm2. This model does not exclude other pathways from contributing to p53 activation by PML. For example, de-ubiquitination of p53 by the herpesvirus-associated ubiquitin-specific protease (HAUSP), which leads to p53 accumulation (46). Although HAUSP is localized to the PML-NBs, its link to PML-mediated p53 regulation is yet to be demonstrated. Further, the acetylation of p53 at Lys-382, which is induced by PML (16), inhibits the ubiquitination of p53 by Mdm2 (47). Thus, it is likely that these effects on the ubiquitination of p53 contribute to counteract the inhibitory effect of Mdm2 on p53 by PML.

Overall, our study is consistent with the notion that the PML-NBs serve as an important meeting point for p53 regulation (48), including phosphorylation (17, 18), acetylation (16), and de-ubiquitination (46). The particular modification(s) involved is dictated by the incoming stress signals. This model is in line with the low frequency of p53 mutations in APL (49). The activation of p53 in APL cells bearing wt p53 may form an attractive therapeutic approach. Treatment of APL patients with all-trans-retinoic acid cures up to 70% of patients. The remaining 30% of patients that relapse are all-trans-retinoic acid-resistant but respond very well to treatment with arsenic trioxide, either alone or in the context of chemotherapy (reviewed in Ref. 50). The efficient activation of p53 in response to low dose arsenic trioxide together with IR (Fig. 4) may provide a rationale for the combined treatment, in particular since adverse effects are associated with high-dose arsenic trioxide treatment (50).

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