Promyelocytic Leukemia Activates Chk2 by Mediating Chk2 Autophosphorylation*

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Chk2 is a kinase critical for DNA damage-induced apoptosis and is considered a tumor suppressor. Chk2 is essential for p53 transcriptional and apoptotic activities. Although mutations of p53 are present in more than half of all tumors, mutations of Chk2 in cancers are rare, suggesting that Chk2 may be inactivated by unknown alternative mechanisms. Here we elucidate one such alternative mechanism regulated by PML (promyelocytic leukemia) that is involved in acute promyelocytic leukemia (APL). Although p53-inactivating mutations are extremely rare in APL, t(15;17) chromosomal translocation which fuses retinoic acid receptor (RAR) and PML is almost always present in APL, while the other PML allele is intact. We demonstrate that PML interacts with Chk2 and activates Chk2 by mediating its autophosphorylation step, an essential step for Chk2 activity that occurs after phosphorylation by the upstream kinase ATM (ataxia telangiectasia-mutated). PML/RARα in APL suppresses Chk2 by dominantly inhibiting the autophosphorylation step, but inactivation of PML/RARα with all-trans retinoic acid (ATRA) restores Chk2 autophosphorylation and activity. Thus, by fusing PML with RARα, the APL cells appear to have achieved functional suppression of Chk2 compromising the Chk2-p53 apoptotic pathway.

Tumor suppressor PML4 that is involved in acute promyelocytic leukemia (APL) mediates multiple apoptotic pathways (1), and acts as an upstream regulator and downstream activator of p53 (2, 3). The proapoptotic Chk2 kinase, whose substrates include p53, Cdc25A, E2F1, PML, and BRCA1, is a key signal transducer to mediate DNA damage signaling (4). Chk2 is activated mainly by ATM in response to DNA damage of double-strand breaks. Chk2 has been shown to be essential for p53 transcriptional and apoptotic activities suggesting the possible cooperation between PML and Chk2 in the p53-dependent apoptosis. Consistent with these results, PML-deficient and Chk2-deficient mice are resistant to γ irradiation-induced apoptosis and prone to cancer after challenge with chemical carcinogens (1, 5). In addition, PML is phosphorylated by Chk2 after γ irradiation, and this phosphorylation was shown to be important for the induction of p53-independent apoptosis (6).

APL is a severe hematopoietic malignancy characterized by a differentiation block of the myeloid progenitor cells at the promyelocytic stage (7–9). The translocation of PML with RARα accounts for nearly all APL cases. PML/RARα acts as a double dominant-negative oncoprotein, inhibiting the RARα and PML pathways (10). PML/RARα is a constitutive transcriptional silencer that blocks RARα-dependent expressions. This interference of the RARα pathway by PML/RARα is an essential component of the differentiation block. Consistent with that, targeted inactivation of PML/RARα upon treatment with all-trans retinoic acid (ATRA) results in terminal differentiation of APL blasts and a transient remission in patients (11). Recent studies suggest that a gain of function of the PML/RARα protein is also an essential feature for leukemogenesis (12, 13).

However, inappropriate expression of dominant negative RARα mutants that cannot interfere with PML function suffices to block myeloid differentiation without causing malignant transformation (14). Furthermore, inactivation of PML leads to an early onset and an increase in the incidence of leukemia in the hCG (human Cathepsin G)-PML/RARα transgenic/PML−/− mice (15, 16). These results suggest that interference of the PML pathway is also of critical importance in the pathogenesis of APL (10, 17, 18).

The PML-mediated apoptosis after DNA damage could be an important checkpoint in maintaining the integrity of genome. In APL cells with PML/RARα, apoptosis is inhibited, at least in part, because of the dominant negative action of PML/RARα over PML (19). These results suggest that disruption of DNA damage-induced apoptosis may play an essential role in initiation and progression of this disease (2, 19). The similarity and shared property of inducing tumor suppression and apoptosis after DNA damage of PML and Chk2 prompted us to investigate the possible functional relationship between...

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4 The abbreviations used are: PML, promyelocytic leukemia; NB, nuclear bodies; APL, acute promyelocytic leukemia; ATM, ataxia telangiectasia-mutated; ATRA, all-trans retinoic acid; RAR, retinoic acid receptor; FL, full-length; hCMV IE1, human cytomegalovirus immediate early 1; WT, wild-type; PBS, phosphate-buffered saline; FACS, fluorescent-activated cell sorter; GST, glutathione S-transferase; Gy, Gray; GFP, green fluorescent protein; HA, hemagglutinin.

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PML is Required for Chk2 Activation

PML and Chk2 in DNA damage response. We report here that PML activates Chk2 by facilitating its autophosphorylation step, an essential step for Chk2 activity that occurs after phosphorylation by the upstream kinase ATM.

EXPERIMENTAL PROCEDURES

Mice and Cells—129Sv PML−/− mice (19)(Pandolfi PP, Sloan-Kettering, NY) and 129Sv/C57BL/6 Chk2−/− mice (Motoyama N. Aichi, Japan) were used in this study. MEFs were prepared from embryos at day 13.5 of development. Early passage MEFs (<5) were used in all experiments. Splenocytes or thymocytes were prepared from 6–8-week-old mice.

Anti-Chk2 Antibodies—Polyclonal rabbit anti-Chk2 (FL) antibody (20) was raised in our laboratory against the GST-tagged human Chk2 recombinant protein expressed using the yeast expression system. In addition to the anti-Chk2 (FL) antibody, we have tested specificity and sensitivity of the following antibody (20) was raised in our laboratory against the GST-antibody and anti-Chk2 (C-18, SC-8813, Santa Cruz Biotechnology, goat polyclonal), anti-Chk2 (A-11, SC17747, Santa Cruz Biotechnology, mouse monoclonal), anti-Chk2 (H-300, SC-9664, Santa Cruz Biotechnology, rabbit polyclonal), anti-Chk2 (Cat. Cell Signaling, rabbit polyclonal), anti-Chk2 (Abcam, mouse monoclonal), and anti-Chk2 (Upstate, mouse monoclonal).

Plasmid Constructs—The bacterial expression vectors for strep-tagged human Chk2 proteins were constructed by PCR or site-directed mutagenesis of Chk2 cDNA. The resulting Chk2 DNA fragments were subcloned into EcoR1/Xhol-digested plasmid pASK IBA7 (Sigma Genosys). Expression vectors for GST-PML proteins, HA-PML IV and V5-Chk2 have been described elsewhere (6). Mammalian expression vectors for HA-PML IV mutant proteins contain nuclear localization signal.

Bacterial Expression and Binding Assay—The solid phase binding assay (Fig. 3a) between Chk2(FL) and GST-PML(FL and fragments) was performed as described (6). The solution binding assays shown in Fig. 3c, the strep-tagged Chk2 proteins were incubated with GST-PML IV extracts (100 ul) or GST tag control extract (100 ul) in binding buffer at 4 °C, overnight. GST-PML proteins were immunoprecipitated by using mouse monoclonal anti-GST antibody (Cell Signaling) and sheep anti-mouse Dynabeads (Dynal). The immunoprecipitate was analyzed by SDS-PAGE and immunoblotted with anti-GST antibody and anti-Chk2 (FL) antibody.

Co-immunoprecipitation Assay—Transfected cells were lysed in cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5 and 1% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Science), and cell extracts were precleared by incubating them with protein A-agarose beads for 2 h at 4 °C. The immunoprecipitating anti-HA or anti-V5 antibodies were conjugated with protein A-agarose beads in cell lysis buffer for 2 h at 4 °C. The antibody-protein A-agarose was collected by brief centrifugation and incubated with cell extracts (100 μg) overnight at 4 °C. The precipitates were washed four times with the cell lysis buffer and dissolved in SDS sample buffer for SDS-PAGE.

Immunoprecipitation Kinase Assay—Endogenous Chk2 was immunoprecipitated with the anti-Chk2 antibody (H-300, Santa Cruz Biotechnology) or anti-Chk2 (FL), and 5 μl of protein A-agarose beads in 150 μl of total volume for 2 h at 4 °C after preclearing the lysates with protein A-agarose beads overnight. For the kinase reaction, GST-PML fragment 1 and GST-Cdc25c (amino acids 200–256) were expressed and purified. Kinase reactions were performed as described previously (6).

Phosphospecific Antibodies and Immunoblotting—Chk2-specific phosphopeptides (Thr68-P; CGTLSLSTVpTQELY (21); Thr383/387-P; KSLRRpTLCGpTPTY) were coupled to keyhole limpet hemocyanin (Pierce). The phosphospecific antibody was purified by two-step purification method, peptide-specific affinity purification step using affinity gel coupled with phosphorylated peptide and peptide-specific affinity absorption step using affinity gel coupled with unphosphorylated peptide (Zymed Laboratories Inc.). Immunoblotting of Chk2 was performed with phosphospecific antibodies (1:100 dilution) after preadsorption of the antisera with 1 mg of 293T cell lysate overnight.

Confocal Microscopy and Detergent Extraction—Cells were grown on coverslips and treated as indicated. The cells were washed in PBS and fixed for 30 min with 4% formaldehyde in PBS with 0.1% Triton X-100. For detergent extraction, cells were extracted before fixation for 5 min at 4 °C in extraction buffer (22) (25 mM HEPES at pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 3 mM magnesium chloride, and 300 mM glucose) containing 0.5% Triton X-100. After five PBS washes, the cells were permeabilized with 0.5% saponin at room temperature for 30 min, followed by two PBS washes. The cells were blocked with 1% chick egg albumin (A-5503, Sigma) in PBS, 0.5% Nonidet P-40 at room temperature for 30 min, followed by two PBS washes. After the wash, the cells were incubated with monoclonal anti-human PML (PG-M3, SC-966, 1:100 dilution, Santa Cruz Biotechnology), polyclonal anti-Chk2 (FL) or anti-Chk2 (H-300, SC-9064, 2 μg/ml, Santa Cruz), polyclonal anti-HA (SC-805, 1:100 dilution, Santa Cruz Biotechnology) or monoclonal anti-V5 antibodies (1:500 dilution, Invitrogen) at 4 °C overnight. After PBS washes, the cells were incubated with Texas Red-labeled AffiniPure Donkey anti-mouse IgG (H + L) (1:600 dilution, overnight incubation) (Cat. No. 715-075-150, Jackson ImmunoResearch Laboratories) or FITC-labeled AffiniPure Donkey anti-rabbit IgG (1:600 dilution in PBS, overnight incubation) (Cat. No. 711-095-152, Jackson ImmunoResearch Laboratories). After washing extensively in PBS and 0.5% Nonidet P-40, cells were further washed in water and mounted in Vectashield mounting medium (Vector Laboratories). Images were captured using a Leica SP confocal microscope.

Retroviral Expression of IE1—Maloney murine leukemia virus-based retroviral vector pLXSN was used to clone the hCMV IE1(WT) gene (a kind gift from Dr. Dejean A, Institut Pasteur, Paris, France and Dr. Ahn JH, Samsung Biomedical Research Center, Seoul, Korea). Retroviral gene transfer experiments were performed as described (23). Briefly, pLXSN-IE1 was transfected into the ecotropic Phoenix packaging cell line (BD Biosciences). Two days later, conditioned media containing the virus was used to infect amphotropic Phoenix packaging.
One day after infection, the packaging cells were split into medium containing G418 (400 μg/ml). After 2 weeks, G418-resistant colonies were picked and expanded in medium with 200 μg/ml G418, and the supernatant viral particles were collected. HeLa cells were transduced overnight with IE1 virus and empty vector virus in the presence of 4 μg/ml of Polybrene. After 48 h, the cells were selected in G418 (200 μg/ml) for about 10 days.

RESULTS

The majority of PML is localized within the nuclear matrix-associated structures known as PML nuclear bodies (NBs), but also is found in the soluble fraction of the nucleus and in the cytoplasm (24). In addition, PML III isoform was reported to be colocalized to centrosomes (25). PML NBs were found to contain proteins involved in multiple cellular processes such as apoptosis, DNA repair, transcription regulation, DNA replication, and RNA transport. Although PML NBs and NB-associated proteins are easily detected by immunofluorescence staining, for most NB-associated proteins, only a subfraction of the protein is thought to be concentrated in NBs (24).

In our previous study, we have observed that a subset of endogenous PML and Chk2 interact and a subpopulation of Chk2 colocalizes with PML NBs (6). In co-immunoprecipitation experiments, Chk2 was shown to be released from the PML complex after γ irradiation and this dissociation required γ irradiation-induced phosphorylations of both PML and Chk2 (6). Recently, other studies reported that Chk2 accumulates on the DNA damage sites briefly but is rapidly released from the damage sites (22), or Chk2 may colocalize with PML NBs after γ irradiation (26).

Whereas our previous work (6) suggested that some Chk2 exists in the PML NBs, other studies have detected only diffusely stained Chk2 (22). If PML and Chk2 can interact as suggested in our co-immunoprecipitation assay using commercially available anti-Chk2 antibodies, however, it is likely that PML NBs may contain some Chk2, regardless of whether the PML-Chk2 complexes function outside or within the PML NBs. To clarify the confusion regarding Chk2 localization, we performed immunofluorescence confocal microscopy with several commercially available anti-Chk2 antibodies (see “Experimental Procedures”) as well as our anti-Chk2 antibody (FL), the only one for which full-length recombinant Chk2 was used as antigen (20, 27). We also visualized Chk2 localization after detergent extraction prior to fixation to remove most of soluble nucleoplasmic proteins (28, 29) so that the Chk2 in the PML NBs could be better visualized.

Chk2 staining in human cell lines was not detectable with most anti-Chk2 antibodies tested (not shown). Only anti-Chk2 (FL) (Fig. 1a) and anti-Chk2 (H-300, Santa Cruz Biotechnology) (6) (supplemental information, Fig. S1) enabled detection of measurable immunofluorescence signal in several human cell lines (not shown) including HeLa cells (Fig. 1a). Anti-Chk2 (FL) showed Chk2 foci as well as diffuse nuclear staining (Fig. 1a), and more than 50% of the NBs showed positive staining with anti-Chk2 (FL) in several human cell lines. Whereas the lot of anti-Chk2 (H-300, lot 1) antibody used in our previous study (6) showed staining patterns similar to that of anti-Chk2 (FL), the current lot of the H-300 antibody (lot 2) showed mostly diffuse nuclear staining reflecting a lot-to-lot variation (supplemental Fig. S1).
Anti-Chk2 (FL) and anti-Chk2 (H-300, lot 1 and lot 2) did not detect signal in HCT15 cells that contain normal PML bodies (6) but no Chk2 (Fig. 1 and supplemental Fig. S1). It is not clear why Chk2 foci are detected only by anti-Chk2 (FL) antibody but it may be due to the fact that anti-Chk2 (FL) was generated against the full-length Chk2 protein while other antibodies were made against peptides or fragments of Chk2 protein. Unlike Chk2 in nucleoplasm, Chk2 complexed to other proteins in the PML NBs may have many of its epitopes hidden and therefore inaccessible to many Chk2 antibodies.

Immunoblotting analysis showed that anti-Chk2 (FL) is highly Chk2-specific. The nonspecific activity of anti-Chk2 (FL) was generated against the full-length Chk2 protein while other antibodies were made against peptides or fragments of Chk2 protein. Unlike Chk2 in nucleoplasm, Chk2 complexed to other proteins in the PML NBs may have many of its epitopes hidden and therefore inaccessible to many Chk2 antibodies.

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associated proteins, transiently expressed V5-tagged Chk2 (V5-Chk2) displayed a diffuse pattern (Fig. 2a). Other studies have also shown a diffuse nuclear staining of Chk2 when overexpressed (20, 22). As expected, HA-PML (PML IV isoform) expressed by transient transfection localized in NBs. However, when V5-Chk2 and HA-PML were expressed together, they colocalized in NBs. The morphology and size of the PML NBs formed by ectopically expressed PML IV could be different from those of endogenous PML NBs containing various isoforms of PML at the physiological level.

Previous studies have shown that the region of PML required for NB targeting is the RBCC region (amino acids 1–520) that consists of three cysteine-rich regions referred to as the RING finger, the B-box (B1/B2) as well as an α-helical coiled-coil (C/C) region (31). Unlike the full-length HA-PML IV protein, PML mutants containing a deletion in the RING, B-box or coiled-coil region did not form NBs (Fig. 2b, left panel). Deletion of the B1- or B2-box individually gave the same result (not shown). V5-Chk2 coexpressed with these non-NB forming HA-PML mutants (Fig. 2b, right panel) also did not localize in NBs. Experiments using HA-PML mutants containing a deletion of the B1- or B2-box individually gave the same results (not shown). In contrast, the HA-PML mutant containing the C-terminal deletion (amino acids 521–633) formed NBs and also recruited V5-Chk2 to these NBs indicating that PML IV can recruit Chk2 into the PML NBs upon transient transfection and the Chk2 targeting domain in PML resides within the RBCC domain.

To determine the Chk2-interacting domain of PML, we performed pull-down experiments using recombinant Strep-Chk2 (FL, WT) and immobilized GST fusion proteins containing various fragments of PML (1–4) (1, amino acids 1–130; 2, amino acids 128–379; 3, amino acids 373–527; 4, amino acids 521–633) (Fig. 3a). Chk2 bound to GST-PML fragments 2 and 4 but not to fragments 1 or 3. We then performed co-immunoprecipitation experiments with transiently expressed V5-Chk2 proteins and various HA-PML mutants containing deletions in the RING-, B1-, B2-, coiled-coil- or C-terminal regions in 293T cells (Fig. 3b). V5-Chk2 co-immunoprecipitated with all of the HA-PML mutants except the B2-box deletion mutant suggesting that the B2-box region of PML is the most critical Chk2-interacting domain.

To determine the Chk2-interacting domain of PML, we next performed in vitro binding experiments with deletion
In APL blasts, which express PML/RARα, NBs display a dispersed microspeckle pattern (32). The PML/RARα fusion protein disrupts the structure and integrity of PML NBs by heterodimerizing with PML and causing dispersion of both PML and PML/RARα into microspeckles. The PML/RARα fusion protein retains most of the functional domains of the parental PML and RARα. The first 59 amino acids of RARα are missing in the fusion protein but the retinoic acid binding and DNA binding domains are intact. As a result, a pharmacological dose of ATRA releases the PML/RARα-mediated transcriptional silencing and causes the APL cells to terminally differentiate. Also, when APL cells are treated with ATRA, PML/RARα is degraded and PML and other NB proteins are relocalized to the NBs.

Because PML is critical for NB formation, we then studied the effect of PML/RARα on Chk2 localization and activity in NB4 APL cells. As described previously, treatment of the NB4 cells with ATRA restored the PML NBs (Fig. 4a) and degraded PML/RARα (not shown). Confocal microscopy revealed that Chk2, like PML, is dispersed in NB4 cells, but a subset of Chk2 is relocalized to the PML NBs after ATRA treatment. The observation that Chk2 is dispersed in NB4 cells but relocalized to PML NBs after ATRA treatment led us to investigate whether Chk2 activity is affected in NB4 cells (Fig. 4b) and whether the integrity of PML NBs affects Chk2 activation (Fig. 4c).

Phosphorylation-dependent mobility shift of Chk2, which normally occurs after Chk2 activation, did not occur after low dose γ irradiation (2.5 Gy) in NB4 cells (Fig. 4b). Similar results were obtained using 1 Gy, 1.5 Gy, or 2 Gy of γ irradiation (not shown). The level of Chk2 in NB4 cells detected by immunoblotting was low compared with that of 293T or HeLa cells (not shown). Interestingly, treatment with ATRA restored Chk2 activation after γ irradiation. This was confirmed with an immunoprecipitation-kinase assay using GST-Cdc25c (amino acids 200–256) and GST-PML 1 (amino acids 1–130) fragments as Chk2 substrates (Fig. 4b). Chk2 immunoprecipitated from ATRA-treated NB4 (2.5 Gy) was active but not Chk2 immunoprecipitated from

FIGURE 4. PML is a Chk2 activator and PML/RARα in APL antagonizes the Chk2 activator function of PML. a, subcellular localization of endogenous PML and Chk2 in APL-derived NB4 cell before and after ATRA (all-trans retinoic acid, 1 μM, 2–3 days) treatment. Cells were immunostained with anti-Chk2 (FL) or anti-PML (PG-M3). b, Chk2 mobility shift and immunoprecipitation kinase assay in NB4 cells after the indicated treatments. c, Chk2 activation after a low dose γ irradiation does not require intact PML NBs. HeLa cells were infected with retrovirus vectors for IE1(WT) or an empty vector. Chk2 immunoprecipitation kinase assay was performed before and after γ irradiation. d, indicated PML mutants were transfected into 293T cells with the expression vector for GFP protein and transfected cells were FACS-sorted after 48 h for immunoprecipitation kinase assay. Endogenous Chk2 was immunoprecipitated for the immunoprecipitation kinase assay, e, Chk2 kinase activity in PML+/+ and PML−/− MEFs after γ irradiation. Endogenous Chk2 was immunoprecipitated for the immunoprecipitation kinase assay.

mutants of Strep-Chk2 and GST-PML IV (full-length). Binding to GST-PML IV was observed with the full-length (FL) Chk2 protein and the Chk2 (amino acids 206–533) and (amino acids 1–384) fragments but not with the Chk2 (del. amino acids 210–384) mutant (Fig. 3c). The same binding pattern was seen in co-immunoprecipitation experiments performed with transiently expressed V5-Chk2 mutants and HA-PML suggesting that the amino acids 210–384 region in the kinase domain of Chk2 interacts with PML (Fig. 3d).

Endogenous Chk2 was immunoprecipitated for the immunoprecipitation kinase assay.
untreated NB4 (2.5 Gy) suggesting the possible PML-dependence of Chk2 activation at low doses of γ irradiation. Chk2 in untreated NB4 cells was efficiently activated after a high dose γ irradiation (5–10 Gy, not shown). At high doses of γ irradiation, Chk2 was shown to be activated via redundant ATM-independent pathways (33). These results suggest that PML may not be required for Chk2 activation in the redundant pathways. Alternatively, PML/RARα may increase the threshold level of DNA damage required for Chk2 activation. The results in Fig. 4b suggested that reactivation of PML or restoration of the PML NBs may be important for Chk2 activation in NB4 cells.

Although the restoration of Chk2 activity in NB4 correlated with the PML/RARα-degrading property of ATRA, a possibility still remained that ATRA restored Chk2 activity in NB4 by a mechanism unrelated to PML/RARα degradation. To rule out the latter possibility, we performed Chk2 immunoprecipitation kinase assay using another human promyelocytic cell line, HL60, that does not contain PML/RARα but undergoes differentiation upon exposure to ATRA. Chk2 in HL60 was activated following γ irradiation and this Chk2 activation was not enhanced after ATRA treatment at any concentrations tested (not shown).

We then examined whether the integrity of PML NBs is important for Chk2 activation after γ irradiation (Fig. 4e). In order to disrupt the PML NBs without affecting the levels of PML isoforms or other NB-associated proteins (34), we infected HeLa cells with a retrovirus vector carrying the human cytomegalovirus IE1(WT) (HCMV major immediate-early protein) gene or an empty control vector. As reported in other studies, the PML NBs were disrupted in the IE1(WT)-infected cells, but not in the control cells (not shown). Chk2 staining with anti-Chk2(FL) also showed a dispersed pattern in the IE1(WT)-infected cells (not shown). In co-immunoprecipitation analysis (supplemental information, Fig. S2), PML-Chk2 interaction was observed both in the IE1-infected cells and the control cells prior to γ irradiation, but decreased after γ irradiation suggesting that the PML-Chk2 complexes can be formed outside of the PML NBs. Chk2 activity in the IE1-infected cells (Fig. 4e) was similar to that in the control cells infected with empty vector. These results suggested that PML NB is not an absolute requirement for Chk2 activation, although some Chk2 exists in the PML NBs and PML is able to recruit Chk2 into the PML NBs after transient transfection.

We next tested PML dominant negative mutants including PML/RARα for an inhibitory effect on Chk2 activation to determine the effect of PML on Chk2 activation (Fig. 4d). Expression of PML/RARα or the PML mutant containing deletion in the RING domain which have been shown to inhibit PML function and disrupt PML NBs (35–37) inhibited activation of endogenous Chk2 after low dose γ irradiation. Other PML mutants containing deletions in the B1-, B2-boxes, and the coiled-coiled region also showed an inhibition (not shown). It is not clear whether the inhibitory effect of the B2-box deletion mutant was obtained because of the dominant negative action of this mutant on PML or the lack of interaction with Chk2, or a combination of both. However, Chk2 was activated in cells transfected with the PML mutant containing the C-terminal deletion that does not disrupt the PML-Chk2 interaction and PML NB formation. These results suggested that PML may regulate Chk2 activation following γ irradiation. To confirm the role of PML in Chk2 activation, we examined Chk2 activity in MEFs from PML+/− and PML−/− mice (Fig. 4e). Chk2 was not activated after low-dose γ irradiation in PML−/− cells but, as it was the case in NB4 cells, Chk2 in PML−/− cells was activated after high dose γ irradiation (not shown). Previous studies using Chk2−/− primary cells indicated that Chk2 is essential for expression of p53-induced apoptotic proteins. The mRNA levels of Puma, Bax, and Noxa induced after γ irradiation were significantly lower in PML−/− and Chk2−/− lymphocytes compared with those in the wild-type cells (supplementary information, Fig. S3). Chk2−/− lymphocytes were slightly more resistant to γ irradiation-induced apoptosis than PML−/− lymphocytes (supplementary Fig. S3).

If PML is required for Chk2 activation as our data suggest, what is its role? Previously, we showed that activation of Chk2 after DNA damage occurs in two sequential steps (21). Activation begins with an ATM-dependent phosphorylation within the SQ/TQ-rich region exemplified by Thr68 phosphorylation. This initial phosphorylation event triggers autophosphorylation at Thr383 and Thr387 in the activation loop of the catalytic domain which is essential for the Chk2 activity and the observed phosphorylation-dependent mobility shift. Although autophosphorylation of Chk2 is an essential maturation event required for full enzymatic activity, to date, possible regulatory requirement for additional proteins that controls autophosphorylation of Chk2 has not been demonstrated.

As shown in Fig. 5a, PML is not required for ATM autoactivation (38) or for ATM-dependent phosphorylation of H2AX, a known marker of irradiation-induced DNA damage (39). γ irradiation-induced ATM autophosphorylation was intact in NB4 cells with or without ATRA treatment during the 3-day treatment period (1 day shown in Fig. 5a, 3 days not shown). In Fig. 5b, after low-dose γ irradiation, phosphorylation of Thr383, but not the phosphorylation of Thr387, was observed in NB4 cells. When PML and/or PML NBs were restored with ATRA treatment in NB4 cells, Chk2 autophosphorylation after γ irradiation was restored in these cells. Impaired autophosphorylation was also observed in PML−/− cells (Fig. 5b) indicating that PML is not required for Chk2 phosphorylation by ATM, but is required for the subsequent autophosphorylation after a low level γ irradiation. As expected, Ser-20 phosphorylation of p53 was also impaired in PML−/− cells after low dose γ irradiation (not shown).

To further confirm the requirement of PML in Chk2 auto-phosphorylation, we suppressed PML by overexpressing PML/RARα in 293T cells and observed that Chk2 autophosphorylation was inhibited in these cells (Fig. 5c). Another implication of this finding is that restoration of Chk2 auto-phosphorylation and activity after γ irradiation in ATRA-treated NB4 cells is indeed caused by destruction of PML/RARα and/or restoration of the PML function but not due to other possible effects of ATRA. To be consistent with the
findings in Fig. 4c that the PML-mediated Chk2 activation can occur outside the PML NBs, autophosphorylation of Chk2 (Fig. 5d) and Thr68 phosphorylation (not shown) were still observed in cells infected with IE1(WT).

Although our results suggest that PML interacts with Chk2 and PML plays an important role for Chk2 activation, we currently do not know whether the PML-Chk2 interaction is required for Chk2 activation after γ irradiation, and, which isoform of PML among seven known isoforms (31) is required for Chk2 activation. Whereas PML IV was used in this study, it...
remains unknown whether PML-null cells reconstituted with PML IV (WT) would elicit the expected Chk2 autophosphorylation in the absence of other PML isoforms. Further investigations using PML−/− MEFs reconstituted with various combinations of PML isoforms or PML IV mutants containing point mutations in the Chk2-interacting domain are under way.

DISCUSSION

Our study demonstrates that ATM and PML work in concert to activate Chk2 by controlling two interconnected but separate phospho-regulatory events (Fig. 5e). Chk2, which is presumably phosphorylated by ATM at the site of DNA break (22), appears to require PML for the subsequent autophosphorylation step. Previous studies proposed that Thr68 phosphorylation by ATM increases Chk2-Chk2 interaction and thereby in trans autophosphorylation (40, 41). Although the molecular mechanism by which PML promotes Chk2 autophosphorylation remains unknown, one possibility is that PML may promote the Chk2-Chk2 interaction following Thr68 phosphorylation. Alternatively, PML may induce Chk2 activation by inactivation of the Chk2-specific phosphatases.

The critical importance of the ATM-Chk2 pathway for cancer progression and cancer prevention has been indicated in recent studies (42, 43). These studies reported that human preneoplastic lesions from a variety of different human cancers express markers of an activated DNA damage response, including phosphorylated ATM, Chk2, p53, and H2AX. Unlike the precancerous or early tumor lesions, late stage tumors showed inactivation of the DNA damage response markers (42, 43), suggesting that disabling the DNA damage response pathways may be a prerequisite for cancer progression and that the activation of the ATM-Chk2 pathway is important for cancer prevention. By demonstrating that ATM and PML work together for Chk2 activation (Fig. 5e), our study introduces a new upstream player into the ATM-Chk2 network suggesting an important regulatory dimension in the DNA damage signaling.

Because p53 activation leads to apoptosis, it has been proposed that the constitutive activation of the upstream components of the ATM-Chk2 pathway in the early stage tumor would provide selective pressure for the high frequency of p53 mutations during tumor progression (44). PML that can function upstream of Chk2 after DNA damage (this study) may then contribute to creating selective pressure for p53 mutations in the early stage tumors. On the other hand, PML/RARα that can induce functional inactivation of Chk2 and p53 may render p53 mutations unnecessary in APL cases (45).

With the additional upstream mode of PML regulation in Chk2 activation shown in this study, we propose that PML may serve as a target for Chk2 inactivation during tumor progression. Consistent with this, a recent study demonstrated a high incidence of PML deficiency in numerous types of tumors (46). PML protein expression was lost in human tumors of multiple histologic origins including prostate, colon, breast, lung, central nervous system, germ cell tumors, and lymphomas. This loss was highly associated with tumor progression and metastatic status in several cancers. The observed loss of PML protein expression or fusion to other proteins such as RARα may abrogate the need for the mutation of Chk2 in a wide range of tumors.

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PML Is Required for Chk2 Activation
