Phosphorylation of PML by mitogen-activated protein kinases plays a key role in arsenic trioxide-mediated apoptosis

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Summary

The promyelocytic leukemia (PML) protein is a potent growth suppressor and proapototic factor, whereas aberrant fusions of PML and retinoic acid receptor (RAR)-α are causal agents in human acute promyelocytic leukemia. Arsenic trioxide (As_2O_3) treatment induces apoptosis in acute promyelocytic leukemia cells through an incompletely understood mechanism. We report here that As_2O_3 treatment induces phosphorylation of the PML protein through a mitogen-activated protein (MAP) kinase pathway. Increased PML phosphorylation is associated with increased sumoylation of PML and increased PML-mediated apoptosis. Conversely, MAP kinase cascade inhibitors, or the introduction of phosphorylation or sumoylation-defective mutations of PML, impair As_2O_3-mediated apoptosis by PML. We conclude that phosphorylation by MAP kinase cascades potentiates the antiproliferative functions of PML and helps mediate the proapoptotic effects of As_2O_3.

Introduction

The promyelocytic leukemia (PML) protein is a RING/B box/coiled-coil (RBCC) domain polypeptide that controls critical aspects of cell cycle progression, senescence, and cell death (Mu et al., 1994; Salomoni and Pandolfi, 2002). Wild-type PML is a potent growth suppressor that, when overexpressed, can block cell cycle progression in a variety of tumor cell lines (Le et al., 1998); conversely, $PML^{-/-}$ mouse embryo fibroblasts, splenocytes, thymocytes, and keratinocytes replicate significantly faster than their $PML^{+/+}$ counterparts, and are resistant to senescence mediated by activated RAS (Le et al., 1998; Liu et al., 1995; Mu et al., 1994; Salomoni and Pandolfi, 2002). PML also plays an essential role in DNA damage- or stress-induced apoptosis, and $PML^{-/-}$ cells are resistant to a variety of apoptotic signals (Wang et al., 1998b). Consistent with these properties, PML can function as a tumor suppressor, and PML disruption leads to elevated incidences of papillomas, carcinomas, and T and B cell lymphomas in several mouse models of oncogenesis (Wang et al., 1998a, 1998b).

In normal cells, the PML protein is localized in, and essential for the biogenesis of, discrete subnuclear compartments variously designated as nuclear bodies (NBs), Kremer bodies, PML oncogenic domains (PODs), or ND10s (Dyck et al., 1994; Ishov et al., 1999; Koken et al., 1994; Weis et al., 1994; Zhong et al., 2000). NBs are sites of protein modification, and at least one function of PML may be to target the p53 tumor suppressor protein to NBs where it can be acetylated, phosphorylated, and activated as a transcription factor (D'Orazi et al., 2002; Fogal et al., 2000; Guo et al., 2000; Hofmann et al., 2002). PML also complexes with and modifies the activity of a wide variety of additional transcription factors, coactivators, and corepressors (Salomoni and Pandolfi, 2002). Intriguingly, NBs are disrupted in human acute promyelocytic leukemias (APL) (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). The most common form of APL arises from a t(15;17) chromosomal translocation that links the N-terminal region of PML to the DNA binding and hormone binding domains of retinoic acid receptor (RAR)-α, a ligand-regulated transcription factor (de The, 1996). The resulting PML-RARα fusion protein is a dominant-negative inhibitor of RARα activity that interferes with transcription of RARα target genes at physiological concentrations of all-*trans* retinoic acid (ATRA); pharmacologically elevated levels of ATRA, however, can overcome the PML-RARα-mediated transcriptional block and can induce terminal differentiation of the APL cell (Grignani et al., 1993a). The ability of PML-RARα to interfere with RARα-signaling clearly plays an important role in the pathogenesis of APL (Brown et al., 1997; Jansen et al., 1995; Lavau and Dejean, 1994). However, PML-RARα is also a dominant-negative inhibitor of the antiproliferative, proapoptotic effects of the native PML progenitor, and these antiapoptotic functions of PML-RARα also contribute to the etiology of APL (de The, 1996; Kawasaki et al., 2003; Rego et al., 2001). Virtually all treatments that cause reversion of APL cells also cause restoration of the NBs, underscoring the importance of the integrity of

SIGNIFICANCE

The PML protein functions as a tumor suppressor in a variety of experimental model systems. Conversely, t(15;17) chromosomal translocations result in aberrant PML expression and are associated with acute promyelocytic leukemia. Notably, these leukemias respond to treatment with As_2O_3, which induces apoptosis of the leukemic cells in culture and disease remission in patients. The mechanism by which As_2O_3 induces apoptosis is incompletely understood. We report here that As_2O_3 treatment induces a MAP kinase cascade that phosphorylates PML and enhances the proapoptotic properties of this protein. Our work provides new insights into the regulation of PML by phosphorylation, and new information about the therapeutic mechanisms of As_2O_3.

NBs to tumor suppression (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994).

Recently, it has been reported that NBs formation requires PML to be conjugated to SUMO (small ubiquitin-related modifier)-1 (Ishov et al., 1999; Zhong et al., 2000). SUMO-1 is an 11 kDa protein that is structurally homologous to ubiquitin (Kim et al., 2002). Sumoylation is thought to regulate the subcellular localization, stability, DNA binding, and/or transcriptional ability of its target proteins such as PML, Ran GTPase activating protein (RanGAP)1, SP100, IκBα, heat shock transcription factor (HSF)2, p53, and c-*jun* (Desterro et al., 1998; Goodson et al., 2001; Gostissa et al., 1999; Mahajan et al., 1998; Matunis et al., 1998; Muller et al., 2000, 1998; Rodriguez et al., 1999; Sternsdorf et al., 1997). Intriguingly, PML sumoylation is altered in response to arsenic trioxide (As_2O_3), a chemotherapeutic agent that is used clinically in the treatment of APL (Everett et al., 1999; Muller et al., 1998). As_2O_3 can induce prolonged remission even in patients with APL refractory to RA treatment and conventional chemotherapy (Niu et al., 1999; Shen et al., 1997). As_2O_3 induces both a partial differentiation and a potent apoptosis in APL cells (Chen et al., 1997); the latter is believed to play a dominant role in the As_2O_3-induced remission of APL (Chen et al., 1997; Zhang et al., 2001). As_2O_3 treatment induces increased sumoylation of PML and restoration of NBs in APL cells, and this sumoylation-associated targeting of PML into NBs may be a mechanism for As_2O_3-induced apoptosis (Muller et al., 1998; Zhu et al., 1997). However, the molecular basis by which As_2O_3 triggers PML sumoylation has not been clarified.

As_2O_3 is known to activate a large number of signal pathways including all three mitogen-activated protein kinases (MAPKs) pathways, i.e., extracellular-signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases pathways (Bode and Dong, 2002). We have previously demonstrated a role of MAP kinase signaling in As_2O_3-induced apoptosis and differentiation of NB4 cells through the phosphorylation of silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). SMRT is an important corepressor for PML-RARα and is essential for the dominant-negative transcriptional properties of this fusion protein; phosphorylation of SMRT leads to its dissociation from its nuclear receptor partner, including RARα and PML-RARα, and a release from transcriptional repression (Hong et al., 2001). Here, we demonstrate that activation of MAP kinase cascades by As_2O_3 has additional effects resulting in the phosphorylation of PML and that this enhanced phosphorylation is associated with increased sumoylation in vivo and may be crucial for PML-dependent apoptosis induced by As_2O_3. This work provides new insights into the therapeutic mechanisms of As_2O_3 treatment.

Results

PML is phosphorylated by the MAP kinase ERK2 in vitro

To test whether PML can be phosphorylated in response to MAP kinase signaling, we performed an in vitro kinase assay using GST-PML as a substrate. The MAP kinase ERK2 efficiently phosphorylated PML under these conditions, whereas MEKK1, SEK1, MEK1, and p38 did not (Figure 1A). In control experiments, all kinases tested exhibited strong activity toward their known substrates (Figure 1B). Use of a series of PML subdomains in the in vitro ERK2 kinase assay indicated that PML was phosphorylated at both N- and C-terminal regions (data not shown). Inspection of the PML sequence in these regions revealed the presence of multiple MAP kinase consensus sites (of the sequence S/T-P; Figure 2A); we therefore introduced a series of alanine substitutions at these sites to map the actual sites of ERK2 phosphorylation. Within the N-terminal domain of PML, individual substitutions of alanine at codons 28, 36, 38, or 40 each measurably reduced phosphorylation of PML by ERK2, whereas the alanine substitution at codon 8 did not (Figure 2B, top panel, and quantified, bottom panel; mutations are designated by the codon number followed by an "A"). All PML constructs were assayed at comparable protein concentrations (Figure 2B, middle panel). These results indicate that multiple positions within the N terminus of PML are phosphorylated by ERK2. Consistent with this proposal, mutations incorporating two or more of these alanine substitutions further impaired phosphorylation relative to the individual mutations (Figure 2B and data not shown), and disruption of all four sites, 28·36·38·40A, virtually abolished phosphorylation of the PML N-terminal domain (Figure 2B). Phosphorylation of the C-terminal domain of PML by ERK2 was measurably reduced by alanine substitutions at codons 527 or 530, but not at codons 505 or 518 (Figure 2C); the combined 527·530A PML mutation virtually eliminated C-terminal domain phosphorylation in vitro by ERK2 (Figure 2C).

We introduced the 28·36·38·40A substitution, the 527·530A substitution, and a combined substitution of both into the full-length PML context and designated these mutants as PML-N, PML-C, and PML-NC, respectively (Figure 2A). These full-length constructs were subjected to the same ERK2 kinase assay as above; all proteins were assayed at comparable concentrations. PML-N and PML-C were phosphorylated to 71% and 40% of the wild-type level, respectively, whereas the combined PML-NC mutant displayed only 10% of the phosphorylation observed for the wild-type PML (Figure 2D). Our results indicate that the principal sites of ERK2 phosphorylation in PML are threonine 28 and serines 36, 38, 40, 527, and 530.

Arsenic trioxide causes phosphorylation of PML through ERK1/2 in vivo

In many cell types, arsenic trioxide is a strong inducer of MAP kinase cascade signaling (Bode and Dong, 2002; Liu et al., 1996). We therefore examined if As_2O_3 could induce the phosphorylation of PML through ERK2. Phosphorylation of proteins can alter their mobility in SDS-PAGE, and we first tested if phosphorylation by ERK2 caused a shift in the mobility of PML. Notably, phosphorylation of wild-type PML by ERK2 in vitro resulted in a reduction in the electrophoretic mobility of PML, phosphorylation of PML-N and PML-C resulted in a more modest, but still detectable mobility shift, and incubation of PML-NC with ERK2 resulted in little or no change in electrophoretic mobility (Figure 3A). These results confirmed that phosphorylation of PML by ERK2 in vitro can be detected as a mobility shift in SDS-PAGE. Next, we examined if As_2O_3 exposure, or coexpression of constitutively active MEK1 (CA-MEK1, an upstream activator of endogenous ERK1/2), resulted in phosphorylation of PML in vivo. Either treatment caused an alteration in the mobility of the wild-type PML protein in SDS-PAGE, and consistent with phosphorylation, this alteration was reversed by alkaline phosphatase (Figures 3B and 3D). Intriguingly, although MEK1 or As_2O_3 signaling enhanced PML phosphorylation, a basal level of phosphorylation of PML was detected even in the absence of specific treatment, manifested as a mobility that was in-

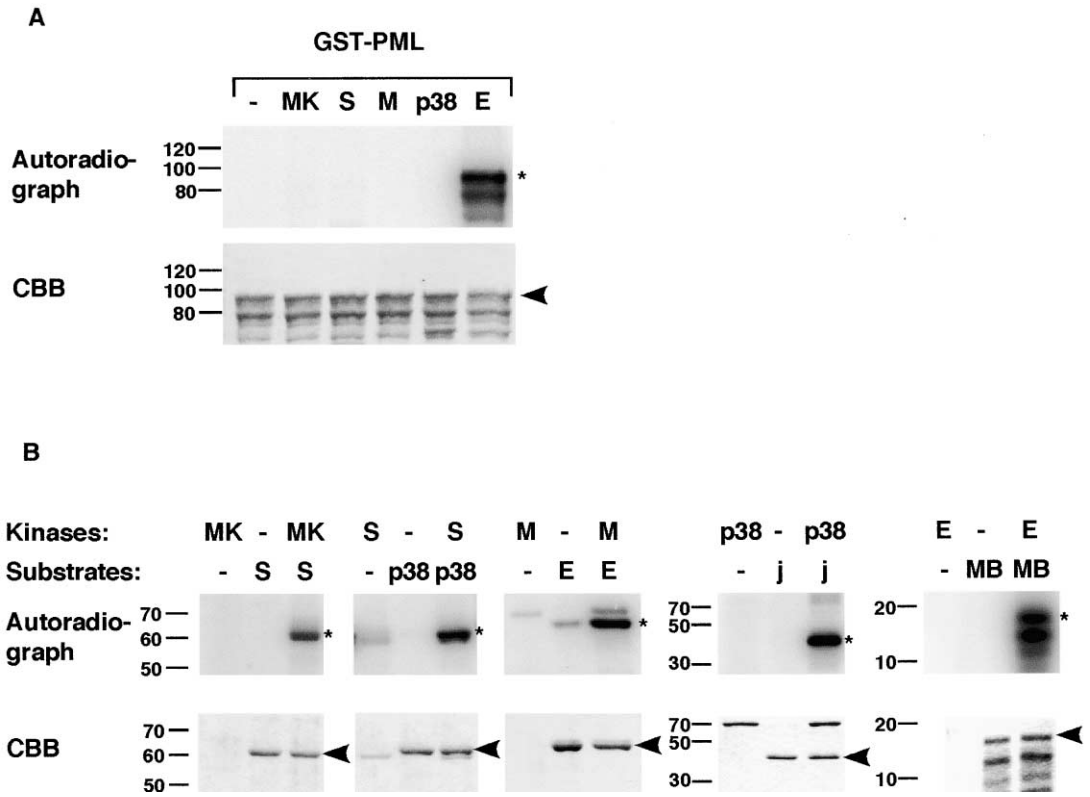

Figure 1. PML is phosphorylated by ERK2 in vitro

A: PML is phosphorylated in vitro. GST-PML was incubated with [γ-^{32}P]ATP and the various kinases indicated. 0.5 μg of active MEKK1 (ΔMEKK1; MK), 0.1 μg of activated GST-SEK1 (S), GST-MEK1 (M), or GST-ERK2 (E), and 0.1 U of activated GST-p38 (p38) were used as kinases; 1 μg of GST-PML was used as substrate. Reaction mixtures were separated by SDS-PAGE. [γ-^{32}P]-incorporation was visualized with a Phosphorimager. The positions of phosphorylated PML and Coomassie brilliant blue (CBB)-stained PML are indicated by an asterisk and an arrow, respectively.

B: Control experiments demonstrate that all enzymes used in **A** were active. 0.5 μg of active MEKK1, 0.1 μg of activated GST-SEK1 (S), GST-MEK1 (M), or GST-ERK2 (E), and 0.1 U of activated GST-p38 (p38) were used as kinases as in **A**; 0.5 μg of unactivated GST-SEK1, GST-p38, or GST-ERK2, and 0.5 μg of c-*jun* (j) or MBP (MB) were used as substrates. Asterisks and arrows indicate phosphorylated substrates and CBB stained substrates, respectively.

creased by alkaline phosphatase treatment (Figure 3B, compare lanes 1 and 2). These results indicate that PML is phosphorylated in proliferating cells, but that inducers of MAP kinases and As_2O_3 signaling further increase PML phosphorylation in vivo.

Consistent with As_2O_3 enhancing PML phosphorylation by activation of a MAP kinase cascade, As_2O_3 treatment increased the phosphorylation/activation of endogenous MAP kinase in these cells, and this phosphorylation was blocked by the MEK1 inhibitor U0126 (Figure 3C; middle panel). Notably, U0126 also blocked the change in mobility of PML in response to As_2O_3 in the same cells (Figure 3C). We conclude that arsenic trioxide activates MEK1 activity and downstream MAP kinases in CHOs, and that the PML phosphorylation induced by As_2O_3 is dependent on an MEK1 pathway. Given that MEK1 fails to phosphorylate PML directly in vitro, it is likely that the As_2O_3-induced phosphorylation of PML in vivo is mediated through the downstream MAP kinases ERK1/2. To confirm this, we examined the effect of our phosphorylation-defective mutations of PML, previously analyzed in vitro, on As_2O_3-induced PML phosphorylation in vivo. Both the PML-N and PML-C mutants exhibited a reduced mobility shift in response to As_2O_3 or CA-MEK1 treatment compared to wild-type PML, and the PML-NC mutant was completely unresponsive to either treatment (Figure 3D). These results indicate that PML phosphorylation in response to As_2O_3 in vivo occurs at the same sites as are phosphorylated by ERK2 in vitro, and support the proposal that As_2O_3 induces PML phosphorylation through activation of ERK1/2. Of note: although the wild-type PML is phosphorylated to some extent even in the absence of the ectopic introduction of MEK1 or As_2O_3, the PML-NC mutant was not (i.e., it migrated at the same mobility plus or minus phosphatase treatment; data not shown). The basal phosphorylation of PML is therefore likely due to the modification of one or more of the ERK1/2 phosphorylation sites.

Phosphorylation of PML in response to arsenic trioxide is associated with enhanced PML sumoylation

Sumoylation alters both the function and the subcellular distribution of PML, and this modification is strongly enhanced by As_2O_3 (Muller et al., 1998). Given that ubiquitination, a related modification, is dependent in many cases on a prior phosphorylation of the protein substrate (Deshaies, 1997; Joazeiro and Weissman, 2000), we investigated if the ability of As_2O_3 to induce PML sumoylation might be mediated through the ability of As_2O_3 to induce PML phosphorylation. We set up an in vivo sumoylation system in which conjugation of a Flag-tagged SUMO to a myc-epitope-tagged PML could be detected by Western analy-

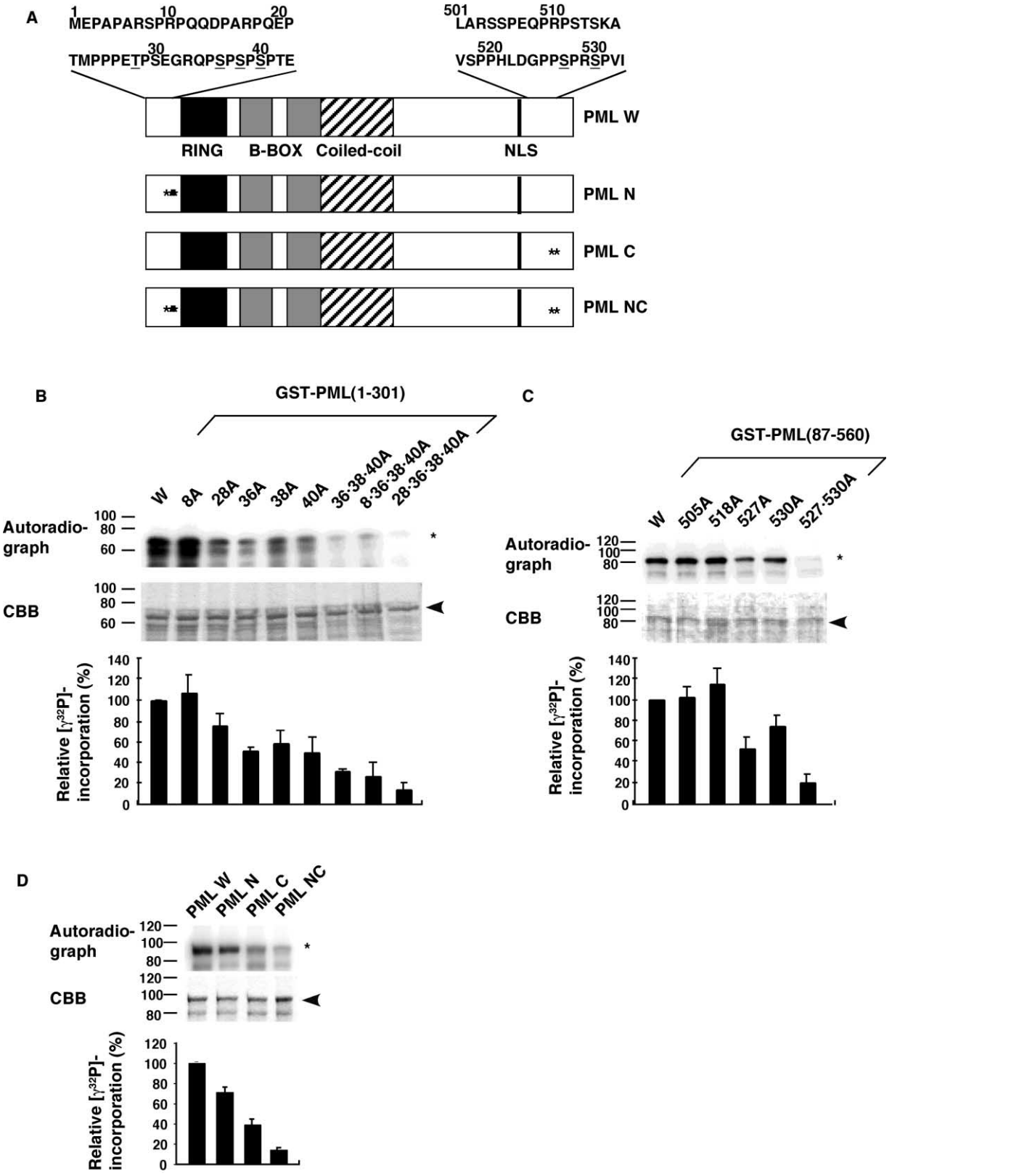

Figure 2. PML is phosphorylated by ERK2 at multiple positions in both N- and C-terminal domains

A: A schematic representation of wild-type and mutant PMLs is shown. The amino acid sequences at potential ERK2 phosphorylation sites are indicated; asterisks indicate the sites of the mutations investigated here. Serines and a threonine likely to be phosphorylated by ERK2 in vitro are underlined.
B: Mutations of potential MAP kinase sites in the PML N-terminal domain reduce phosphorylation by ERK2. Equal amounts (0.5 μg) of a GST-PML (aa 1–301) fusion construct, either wild-type or mutant, were incubated in vitro with ERK2 and were subjected to SDS-PAGE, CBB staining, and autoradiography as in Figure 1. [γ^{32}P]-incorporation into each GST-PML construct was quantified using a Phosphorimager. A representative Phosphorimager scan (top panel), the

sis, resulting in the appearance of a novel 100 kDa PML-SUMO species containing both epitope tags (Figure 4A); as expected, this novel species was not observed if the Flag-SUMO and Ubc9 expression vectors were omitted from the transfections (Figure 4A). Using this system, we examined the role of MEK1 pathway in the As_2O_3-induced sumoylation of PML. As_2O_3 exposure resulted in a significant increase in sumoylaton that began an hour after arsenic exposure and reached a maximum 6 hr later (Figure 4B, top panel). The kinetics of sumoylation paralleled that of ERK 1/2 activation (Figure 4B, third panel) and of PML phosphorylation (Figure 4B, top panel); a lighter exposure is also provided to better visualize the change in electrophoretic mobility of PML in response to phosphorylation (Figure 4B, second panel, at least two phosphorylated PML species can be detected). As_2O_3 treatment increased sumoylation of wild-type PML approximately 2-fold (Figure 4C). Treatment of the CHO cells with the MEK1 inhibitor U0126 abolished this As_2O_3-induced increase in PML sumoylation, whereas the effects of arsenic trioxide were mimicked by expression of the CA-MEK1 construct (Figure 4C). Consistent with these observations, both the PML-N and PML-C mutants displayed a reduced sumoylation in response to either As_2O_3 or CA-MEK1, and the PML-NC mutant exhibited no increase in sumoylation in response to either treatment (Figure 4D). Overall, our experiments indicate that arsenic trioxide induces a MEK1-ERK1/2 pathway resulting in enhanced phosphorylation of PML and an associated increase in the sumoylation of this protein.

PML phosphorylation by ERK1/2 enhances PML-dependent apoptosis in response to arsenic trioxide

To determine the biological significance of PML phosphorylation, we investigated whether the As_2O_3-induced phosphorylation and sumoylation of PML plays a role in apoptosis. PML potentiates the apoptotic effects of tumor necrosis factor (TNF) α and of γ-irradiation (Wu et al., 2003; Yang et al., 2002), and PML might similarly enhance the proapoptotic effects of As_2O_3. We were unable to detect endogenous PML protein in our untransfected CHO cells by immunofluorescence (Figure 5E and data not shown), using antibody that could detect human PML in HeLa cells and rodent PML in Rat 2 cells (Figure 5E). Similarly, little or no PML mRNA was detected in untransfected CHO cells using RT-PCR and primers to PML sequences conserved in human and mouse DNA databases; control PCR experiments using genomic DNA from CHO cells as template confirmed that these primers were capable of efficiently recognizing hamster PML sequences (data not shown). These determinations do not demonstrate that CHO cells are truly PML negative; PML may be expressed in these cells at low levels below the limits of sensitivity of our assays. Nonetheless, we conclude that these cells are suitable for examining the effects of overexpression of ectopically introduced PML and PML mutants.

Notably, overexpression of PML in the CHO cells by transient transfection substantially increased As_2O_3-induced cell death relative to cells transfected with an empty vector (Figure 5A, compare the blue and red dashed lines). Cotransfection of expression vectors for SUMO and Ubc9 further sensitized the PML-transfectants to As_2O_3-induced cell death (Figure 5A, compare the solid and dashed blue lines) whereas the proapoptotic effects of SUMO and Ubc9 were greatly reduced in the absence of PML (Figure 5A, red lines). These results indicate that PML potentiates cell death in response to As_2O_3 and suggest that sumoylation further enhances the efficacy of PML in this context. To test this hypothesis, we created the PML-3K mutant, which has lysine-to-arginine mutations at all three PML sumoylation sites and cannot be sumoylated (Kamitani et al., 1998a). Overexpression of PML-3K in either the presence or absence of SUMO/Ubc9 had little or no effect on As_2O_3-induced cell death (Figure 5A, green lines), indicative of a requirement for PML sumoylation in arsenic-mediated cell death. We next investigated the ability of our phosphorylation-defective mutants of PML to mediate As_2O_3-induced cell death. The PML-N and PML-C mutants displayed an impaired ability to mediate cell death in response to As_2O_3 which was reduced still further in the combined PML-NC mutant (Figure 5B, compare the suppression of cell viability by the PML mutants, light blue, purple, and green lines, respectively, to the wild-type PML, dark blue line). Immunoblotting confirmed that equal levels of PML were expressed for each mutant (data not shown). Similarly, using U0126 to block MEK1 signaling reduced cell death in response to wild-type PML plus As_2O_3 to the levels observed for PML-NC mutant, further suggesting that phosphorylation of PML plays an important role in conferring cell death by As_2O_3 (Figure 5C, compare the dashed and solid lines).

Next, we performed TUNEL assay to determine if the decreased viability/increased cell death mediated by PML and As_2O_3 was due to apoptosis. We obtained essentially the same results by TUNEL assay as for the cell death assay: apoptosis of CHO cells in response to 3 μM As_2O_3 exposure was enhanced by ectopic expression of PML and still further enhanced by coexpression of PML, SUMO, and Ubc9 (Figure 5D, lanes 1–4). Both PML-N mutant and PML-C mutant transfectants exhibited a reduction in this As_2O_3-dependent apoptosis compared to wild-type PML transfectants, and PML-NC mutant displayed the weakest apoptotic activity in this regard (Figure 5D, lanes 4–7). Furthermore, the U0126 MEK1 inhibitor inhibited apoptosis by the wild-type PML to levels approaching those seen with the phosphorylation-defective PML-NC mutant (Figure 5D, lane 4 versus 7, 9, and 10). MEK1 inhibitor did not affect apoptosis in CHO cells transfected with empty vector (Figure 5D, lanes 1 and 8), and these cells displayed little or no apoptosis in the absence of As_2O_3 (data not shown). These results indicate that PML phosphorylation in response to As_2O_3 results in increased sumoylation and increased PML-dependent apoptosis.

corresponding CBB-stained electrophoretogram (middle panel), and the quantitation of the [γ^{32}P]-incorporation for each mutant, relative to the wild-type PML construct (bottom panel), are presented. The average of three independent analyses and standard deviations are shown. Phosphorylated PML and CBB-stained PML are indicated by an asterisk and an arrow, respectively.

C: Mutations of potential MAP kinase sites in the PML C-terminal domain reduce phosphorylation by ERK2. Equal amounts (0.3 μg) of a GST-PML (aa 87–560) fusion construct, either wild-type or mutant, were subject to in vitro kinase assay as in **B**.

D: Mutation of potential ERK2 sites in full-length PML reduces phosphorylation by ERK2. Equal amounts (0.5 μg) of full-length wild-type or mutant PML were subject to in vitro kinase assays as described in **B**.

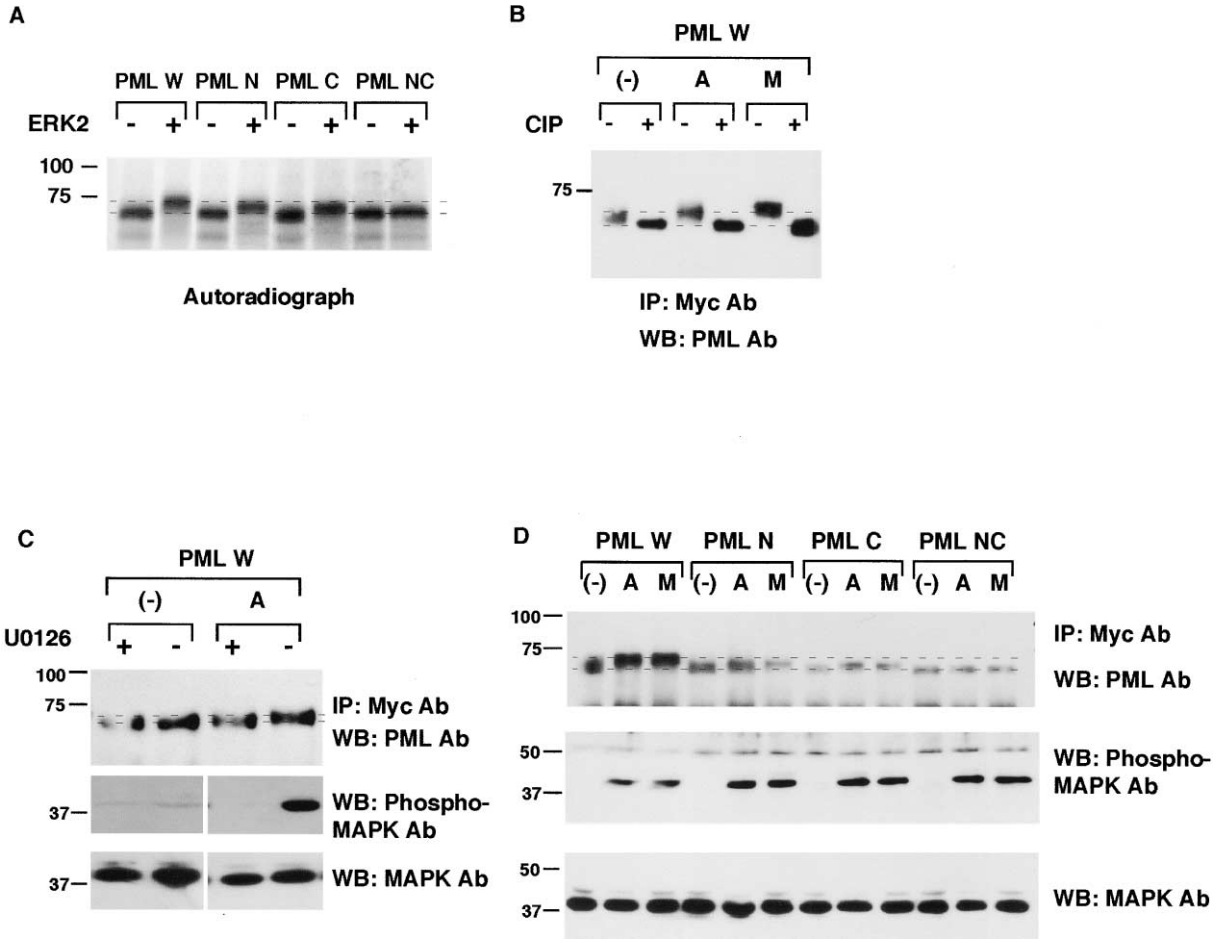

Figure 3. PML is phosphorylated in vivo by a MAP kinase cascade

A: Phosphorylation in vitro alters the electrophoretic mobility of PML. ^{35}S-labeled PML proteins (wt or mutant) were subjected to an in vitro kinase/mobility shift assay using unlabeled ATP in the presence (+) or absence (–) of activated GST-ERK2, were resolved by SDS-8% PAGE, and were visualized by autoradiography. The mobilities of unphosphorylated (lower) and phosphorylated (higher) wild-type PML are indicated by the broken lines.

B: Arsenic trioxide or MEK1 treatment alters the electrophoretic mobility of PML in vivo. CHO cells were transfected with an expression vector for myc-tagged PML and were cultured in the absence (–) or presence (A) of As_2O_3, or were cotransfected with an expression vector for CA-MEK1 in the absence of As_2O_3 (M). The cells were then lysed, the lysates were immunoprecipitated with anti-myc antibody, and the immunoprecipitates were treated (+) or not (–) with calf intestinal phosphatase (CIP) as indicated. The immunoprecipitates were then subjected to SDS-8% PAGE and immunoblotting with anti-PML antibody (H238). The mobilities of phosphatase-treated PML from untreated cells (lower) and untreated PML from CA-MEK1-treated cells (higher) are indicated by the broken lines.

C: U0126 inhibits As_2O_3-mediated modification of PML in vivo. Cells were transfected and treated as described in **B** but in the presence or absence of the MEK1 inhibitor, U0126. Cell lysates were immunoprecipitated with anti-myc antibody, and the immunoprecipitates were subjected to SDS-8% PAGE and immunoblotted with anti-PML antibody (H238, top panel). Whole-cell lysates were also electrophoresed and immunoblotted with anti-phospho-MAPK antibody (middle panel) or anti-MAPK antibody (bottom panel). The mobilities of PML from untreated cells (lower) and from As_2O_3-treated (in the absence of U0126) cells (higher) are indicated by the broken lines.

D: Mutations in PML amino acids subject to phosphorylation in vitro restrict modification of PML in response to As_2O_3 and MEK1 in vivo. Cells were treated as described in **B**. PML mobility and MAP kinase activation were analyzed as described in **C**. The mobility of PML-NC from untreated cells (lower) and PML-W from cells coexpressing CA-MEK1 (higher) is indicated by the broken lines.

Transiently transfected cells often express the transgene at higher than physiological levels. We therefore also established CHO cells stably transfected with wild-type or mutant PML to examine the apoptotic effects of PML when expressed at a more physiological level. Stable transfectants were designated with the name of PML construct transfected; i.e., CHO-W is a stable transfectant of PML-W. PML expression and formation of PML nuclear bodies (NBs) in stable transfectants were confirmed by immunofluoresence with anti-PML antibody. All mutant or wild-type transformants expressed PML and displayed NB formation at levels comparable to that seen for endogenous PML expression in HeLa cells and Rat 2 cells, whereas parental CHO cells did not express PML at a detectable level (Figure 5E and data not shown). We performed cell death assay using these stable transfectants and obtained essentially the same results as those using transient transfectants: CHO-W showed an enhanced sensitivity to As_2O_3-induced cell death relative to parental CHO cells (Figure 5F). Both CHO N and CHO C exhibited a reduction in this As_2O_3-dependent cell death compared to wild-type PML transfectants, and CHO NC displayed the

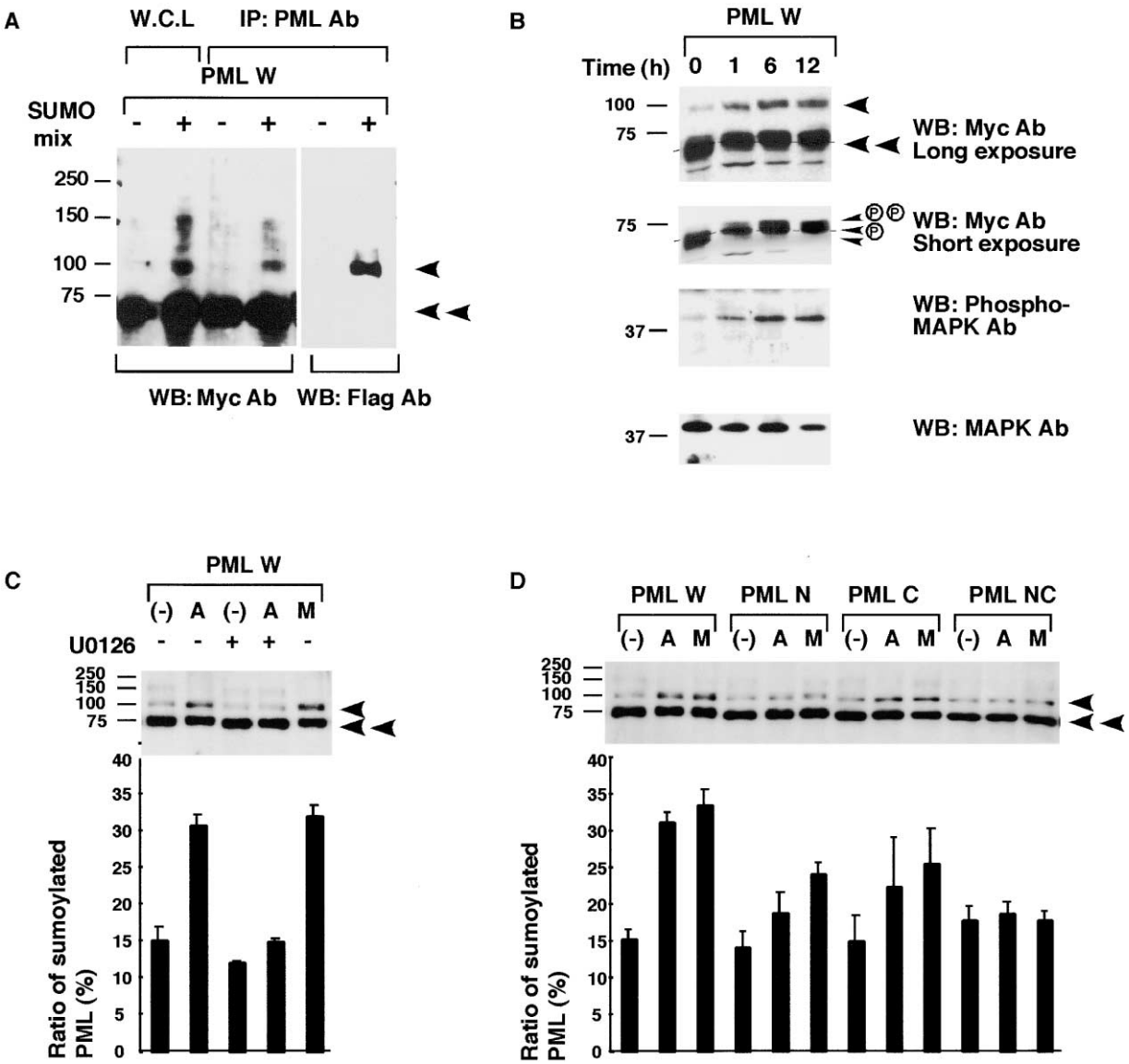

Figure 4. PML phosphorylation by ERK1/2 is associated with enhanced sumoylation

A: PML is sumoylated in vivo. CHO cells were transfected with a pCDNA expression vector for myc-tagged wild-type PML with or without cotransfection of pCMV-Flag-SUMO and pCMV-Flag-Ubc9 (SUMO mix). Whole-cell lysates (W.C.L) were prepared from these cells and either the W.C.L or immunoprecipitates using anti-PML antibody (H238) were subjected to SDS-8% PAGE followed by immunoblotting with anti-myc or anti-Flag antibody. Sumoylated and unsumoylated PML are indicated by single and double arrows, respectively.

B: Kinetics of PML sumoylation and phosphorylation and ERK1/2 phosphorylation coincide. CHO cells transfected with pCDNA-myc wild-type PML, SUMO, and Ubc9 were treated with As_2O_3 for the times indicated and lysed. The lysates were resolved on SDS-8% PAGE and immunoblotted with anti-myc antibody (top panel and, in a shorter exposure, second panel), with anti-phospho-MAPK antibody (third panel), or with anti-MAPK antibody (bottom panel). Sumoylated and unsumoylated PML are indicated by single and double arrows, respectively (top panel). PML with fast, intermediate, and slow mobilities, representing different phosphorylation states, are indicated by an arrow, by a circled P, and by two circled Ps, respectively (second panel). The mobility of PML with intermediate phosphorylation is indicated by the broken lines (top and second panel).

C: PML sumoylation is enhanced by As_2O_3 or MEK1, but inhibited by U0126 treatment. CHO cells were transfected with pCDNA-myc wild-type PML, SUMO, and Ubc9 and were cultured in the absence (−) or presence (A) of As_2O_3, a cotransfected CA-MEK1 expression vector (M), and/or U0126 as indicated. Cell lysates were subjected to SDS-12% PAGE, immunoblotting with anti-myc antibody, and detection with a Fluorchem 8900 Imaging System. A representative image is shown (top panel); sumoylated and unsumoylated PML are indicated by single and double arrows, respectively. The ratio of sumoylated PML to total PML was quantified for three independent analyses, and the average and standard deviations are presented (bottom panel).

D: PML mutations that restrict PML phosphorylation also restrict PML-sumoylation in response to arsenic trioxide and to MEK1. Wild-type and mutant PML constructs were analyzed as in **B**.

weakest cell death activity in this regard (Figure 5F, compare the suppressed cell viability of mutant transfectants, light blue, purple, and green lines, respectively, to the wild-type transfectant, dark blue line). Furthermore, U0126 inhibited cell death of CHO-W to levels approaching those seen with CHO NC (Figure 5G, compare the dashed and solid dark blue lines). These results on stable transformants therefore paralleled our results with the transient transfection system.

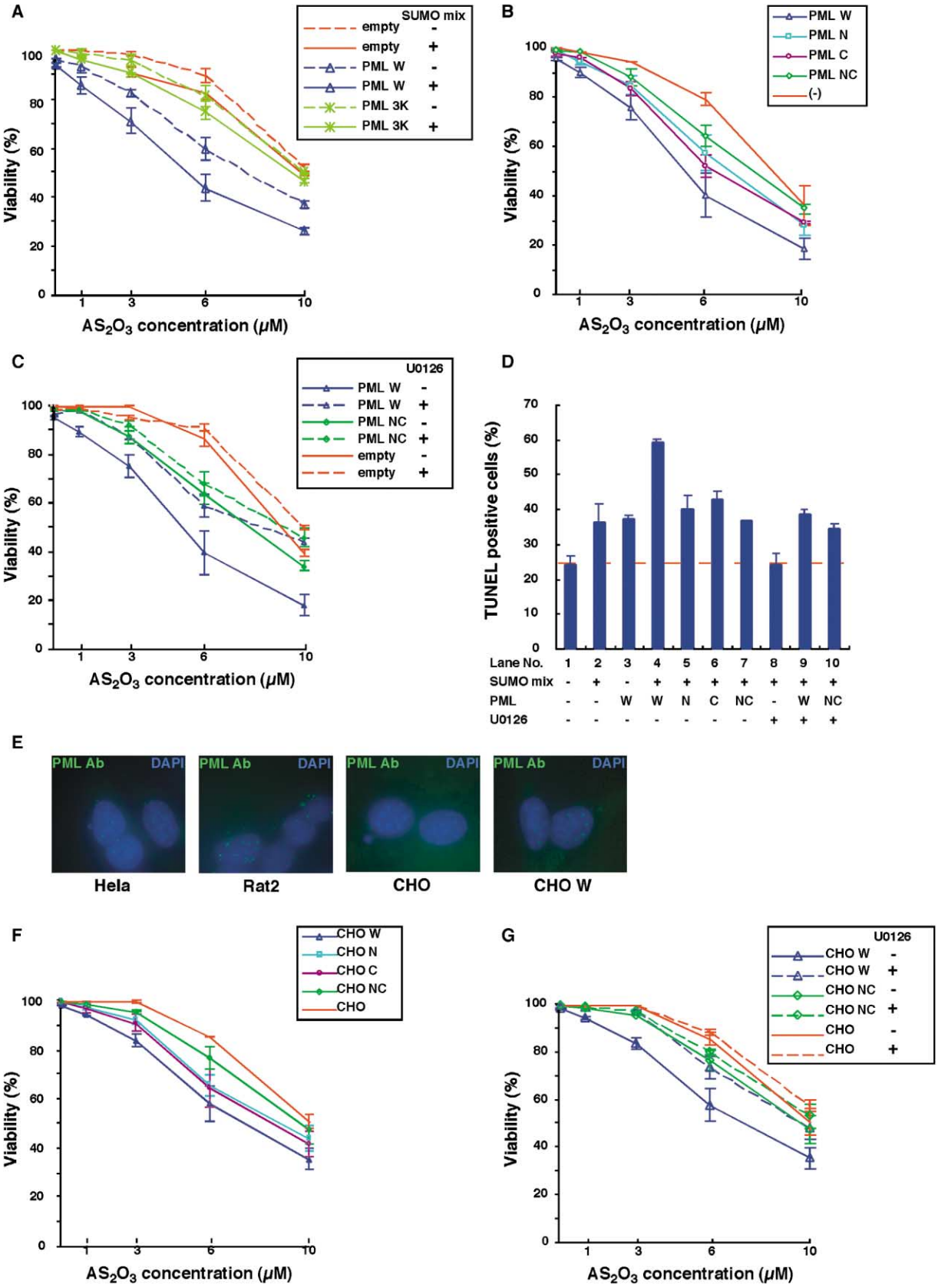

Figure 5. PML phosphorylation at ERK sites plays an important role in As_2O_3-potentiated apoptosis

A: Wild-type PML enhances As_2O_3-induced cell death. CHO cells were transiently transfected with an empty vector, a vector expressing wild-type PML, or a vector expressing the sumoylation-defective PML-3K mutant, and were treated with As_2O_3 for 24 hr at the concentrations indicated below the panel.

PML-RARα is phosphorylated by ERK1/2 in response to arsenic trioxide but does not enhance cell death

The ERK2 phosphorylation sites present in the PML N terminus are retained in the PML-RARα fusion oncoprotein, suggesting that PML-RARα might also be an ERK2 substrate. We examined the in vivo phosphorylation of wild-type PML-RARα (PR W), and of PML-RARα mutants lacking the N-terminal phosphorylation sites found in PML (PR N, Figure 6A). Wild-type PML-RARα exhibited a mobility shift in response to coexpression of CA-MEK1 (Figure 6B); this shift of PML-RARα was less than that of wild-type PML, presumably due to the absence of the C-terminal phosphorylation sites present in the native PML (Figure 6A). This shift in the mobility of wild-type PML-RARα was not observed in the PR N mutant, suggesting that the same N-terminal amino acids identified as phosphorylation sites in PML are also phosphorylated in PML-RARα. As_2O_3 treatment resulted in a greater shift of wild-type PML-RARα than that seen in coexpression of CA-MEK1, and the PR N mutant exhibited a reduced, but still detectable shift in response to As_2O_3 (Figure 6B). These results suggest that PML-RARα is phosphorylated by the ERK1/2 kinase cascade at the N-terminal sites found in native PML, whereas As_2O_3 induces phosphorylation of PML-RARα both at these N-terminal PML sites and probably at additional locations with RARα sequences.

PML-RARα has been reported to be less sensitive to sumoylation than PML (Kamitani et al., 1998a, 1998b) and we could not detect sumoylation of PML-RARα even when SUMO and Ubc9 were coexpressed (data not shown). Given that sumoylation-defective mutants of PML are impaired in mediating apoptosis in response to As_2O_3 (Figure 5A), the proapoptotic effect observed for wild-type PML might be expected to be lost in PML-RARα. Indeed, transient transfection of wild-type PML-RARα into CHO cells caused little or no enhancement of cell death in response to As_2O_3 (Figure 6C).

PML-RARα has been proposed to function, in part, by opposing the proapoptotic effects of PML; acute promyelocytic cells that express both PML and PML-RARα are therefore subject to the opposing actions of both pro- and antiapoptotic forms of PML. In this model, As_2O_3 would be able to overcome the antiapoptotic effects of PML-RARα by phosphorylating PML and enhancing its proapoptotic effects. We therefore performed the cell death assay using an APL-derived cell line, NB4 cells, with or without cotreatment of MEK1 inhibitor. As anticipated, As_2O_3 induced NB4 cell death, whereas the MEK1 inhibitor, U0126, significantly inhibited the cell death induced by As_2O_3, suggesting an important role of MEK1 signaling in the therapeutic treatment of APL with As_2O_3 (Figure 6D).

Discussion

The data presented here demonstrate that phosphorylation of PML by MAP kinases plays an important role in the control of PML-dependent apoptosis in response to As_2O_3 exposure. PML is also involved in apoptosis by Fas, TNFα, ceramide, and type I and II interferons; all of these stimuli activate signaling pathways that include MAP kinases and may operate, at least in part, through the ERK 1/2 phosphorylation events described here (Cuvillier et al., 1996; David et al., 1995; Goillot et al., 1997; Li et al., 2002; Vietor et al., 1993; Wang et al., 1998b). PML is also a substrate for other kinases associated with the control of cell death, either positively or negatively. For example, PML phosphorylation by the DNA damage checkpoint kinase hCds/Chk2 enhances radiation-induced apoptosis (Yang et al., 2002). Mitosis-specific phosphorylation of PML by an unknown kinase(s) has been reported to inhibit PML sumoylation (Everett et al., 1999).

Although the ERK pathway is most often involved in cell survival, proliferation, and differentiation (Cobb, 1999), ERK2 activation has also been reported to mediate certain apoptotic signals, such as those induced by Fas ligand in human neuroblastoma cells, or by transforming growth factor β (TGFβ) in breast carcinoma cells (Frey and Mulder, 1997; Goillot et al., 1997). Radiation-induced apoptosis in human myeloblastic leukemia cells and activation-induced cell death of mouse T cells are also known to operate through ERK2 pathways (Li et al., 2002; Zhu et al., 1999). The contribution of ERK1/2 to apoptosis in these cases was attributed, in part, to increases in the expression of Fas ligand, Fas receptor, or jun D, but much remains unclear as to the mechanisms involved (Goillot et al., 1997; Li et al., 2002; Zhu et al., 1999). Our elucidation that ERK1/2 can phosphorylate PML directly may provide additional insights into these questions.

It should be noted that even though ERK1/2 activation is required for apoptosis induction in response to a number of

"SUMO mix" indicates experiments in which expression vectors for SUMO and Ubc9 were cotransfected together with the PML construct. Cell viability was determined by trypan blue exclusion. The average and standard deviation of two independent duplicate assays are presented.

B: Mutants of PML impaired for ERK2 phosphorylation are also impaired for As_2O_3-induced cell death. Expression vectors for wild-type and mutant PML, or an analogous empty vector (−), were transfected with SUMO mix as indicated. Cell death was analyzed as described in **A**.

C: U0126 inhibits PML potentiation of As_2O_3-induced cell death. Expression vectors for wild-type PML or PML-NC mutant or empty vector were transfected together with SUMO mix. Cells were then treated with As_2O_3 at the indicated concentrations in the presence (+) or absence (−) of U0126 for 24 hr. Cell death was analyzed as described in **A**.

D: Phosphorylation of PML correlates with As_2O_3-induced apoptosis. Expression vectors for wild-type (W) or mutant (N, C, and NC) PML, or an analogous empty vector (−), were transfected with or without SUMO mix. Cells were then treated with 3 μM As_2O_3 in the presence (+) or absence (−) of U0126 for 24 hr. The percentages of apoptotic cells were scored by TUNEL assay; the average and standard deviations of two independent experiments are shown. The broken line indicates the level of TUNEL-positive cells for the empty vector control.

E: Expression of PML stably transfected into CHO cells is comparable to its endogenous expression in HeLa cells and Rat 2 cells. Cells were subjected to immunofluoresence analysis using anti-PML antibody (5E10) with FITC-conjugated secondary antibody, or DAPI. Representative images are shown (PML, green; DAPI, blue).

F: Wild-type PML enhances As_2O_3-induced cell death in stable cell transformants. Stable CHO transfectants expressing the PML alleles indicated were subjected to a cell death assay as in **A**.

G: U0126 inhibits the PML potentiation of As_2O_3-induced cell death in stable transfectants. Stable transfectants of CHO cells were treated with As_2O_3 at the indicated concentrations in the presence (+) or absence (–) of U0126 for 24 hr. Cell death was analyzed as in **A**.

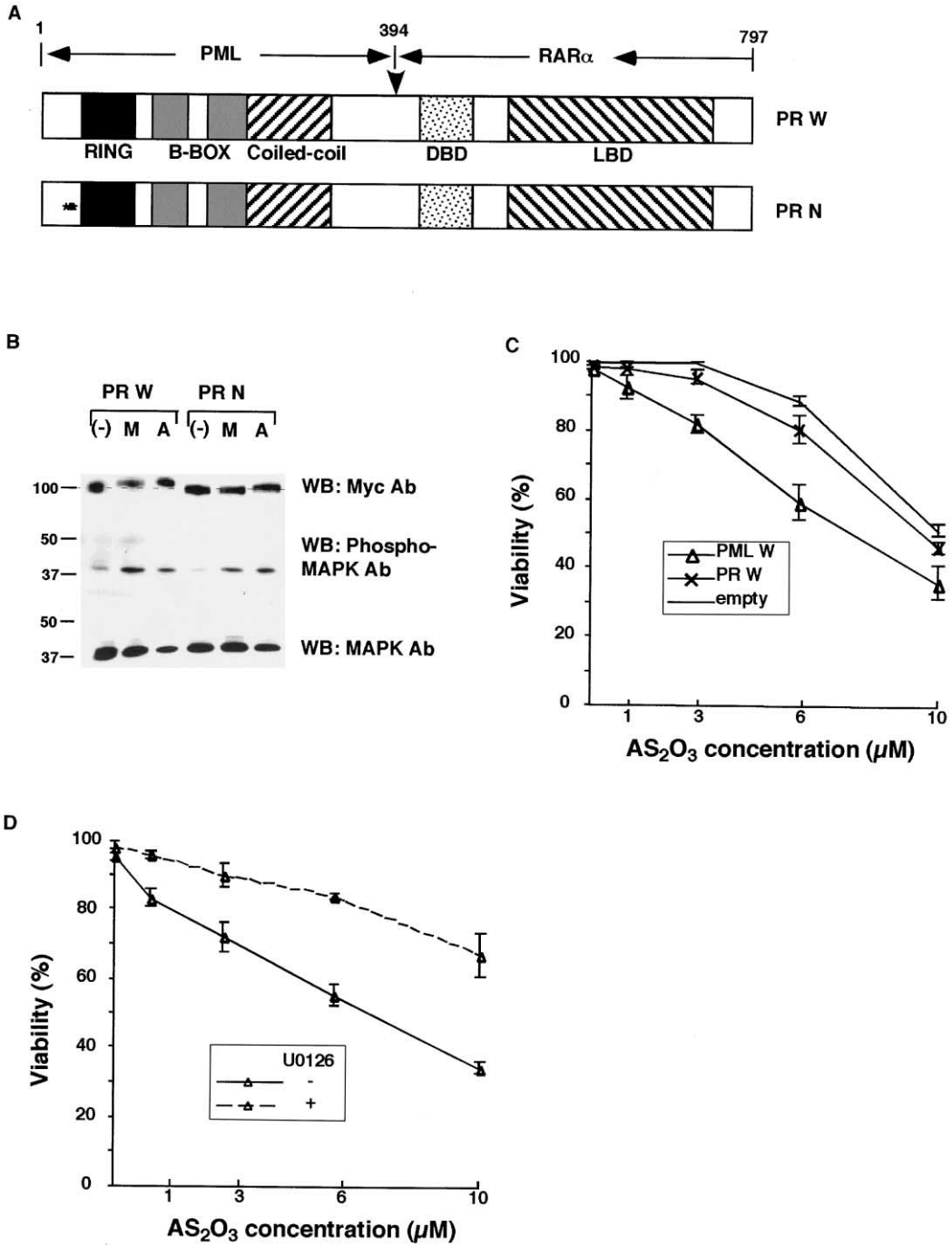

Figure 6. PML-RARα is phosphorylated by ERK1/2 but does not potentiate apoptosis in response to As_2O_3

A: A schematic representation of wild-type and mutant PML-RARα is shown. The DNA binding domain (DBD) and ligand binding domain (LBD) of RARα are indicated. The vertical arrow indicates the translocation breakpoint. Asterisks indicate the position of the mutations created.

B: As_2O_3 and MEK1 induce a mobility shift in PML-RARα. CHO cells were treated as in Figure 3B but using myc-tagged PML-RARα (PR) instead of PML. Changes in the electrophoretic mobility of PML-RARα in response to expression of MAP kinase or arsenic treatment were analyzed as in Figure 4B. The mobility of PR W from untreated cells is indicated by the broken lines.

C: PML-RARα displays little or no ability to potentiate the apoptotic effects of As_2O_3. CHO cells were transiently transfected with an empty vector, the PML vector (PML), or the PML-RARα vector (PR), and were subjected to cell death assay as described in Figure 5A.

D: MEK1 inhibitor inhibits the As_2O_3-induced cell death of NB4 cells. NB4 cells were treated with As_2O_3 at the indicated concentrations in the presence (+) or absence (–) of U0126 for 24 hr. Cell death was analyzed as in Figure 5A.

cell signaling pathways, ERK1/2 activation per se is typically insufficient for apoptosis. This is consistent with our own observation that simple coexpression of PML and CA-MEK1 does not cause apoptosis (F.H. and M.L.P., unpublished data). Since PML phosphorylation appears to be required for PML-dependent apoptosis by As_2O_3, additional signals from As_2O_3 must be necessary for the induction of PML-dependent apoptosis. Other members of MAP kinase family, such as JNK and p38, are

activated in parallel to, and can work cooperatively with, ERK to induce apoptosis (Frey and Mulder, 1997; Goillot et al., 1997; Zhu et al., 1999). Although p38 cannot phosphorylate PML directly in vitro (Figure 1A), p38 or other MAP kinases may be involved in As_2O_3-induced apoptosis by targeting proteins other than PML itself. It should also be noted that parental CHO cells lacking detectable PML expression are still sensitive to apoptosis by As_2O_3, although at a much reduced level compared to cells expressing PML. Either PML may be expressed in our CHO cells at levels too low to be detected, or there may be PML-independent apoptotic mechanisms that respond to As_2O_3. The later possibility is not surprising, considering the variety of signals activated by As_2O_3. Although we employed arsenic trioxide at concentrations (1 to 10 μM) that overlap the plasma levels achieved in the clinical treatment of APL (from 1 to over 6 μM), the mechanisms induced by arsenic trioxide in vivo and ex vivo may also differ.

Sumoylation of PML is necessary for NB formation (Ishov et al., 1999; Zhong et al., 2000), and the apoptotic effects of PML may be dependent on NB formation (see Introduction). Given our findings that PML phosphorylation by ERK1/2 is associated with increased PML sumoylation, this sumoylation-dependent NB formation may represent one of the mechanisms by which PML phosphorylation can enhance apoptosis. This hypothesis is supported by our findings that coexpression of SUMO and Ubc9 enhance PML-dependent apoptosis by As_2O_3, that phosphorylation-defective mutants of PML exhibit defects in sumoylation and apoptosis in response to As_2O_3 treatment, and that sumoylation-impaired mutants of PML, such as PML-3K and PML-RARα, are also defective for NB formation and for As_2O_3-mediated apoptosis.

Phosphatase inhibitors can inhibit PML sumoylation in vivo, suggesting that phosphorylation can negatively regulate sumoylation in some contexts (Everett et al., 1999). The studies we report here, in contrast, suggest that there is a positive control of PML sumoylation by phosphorylation. How might phosphorylation lead to increased sumoylation? ERK-phosphorylated PML might be a better substrate for Ubc9, the E2 conjugating enzyme that transfers SUMO groups onto substrate proteins. We could not, however, demonstrate an increase in PML sumoylation in response to ERK2 in an in vitro modification system (F.H. and M.L.P., unpublished data), suggesting that regulation of sumoylation in vivo involves additional components not present in the in vitro assay. Phosphorylation of PML might regulate PML traffic from nucleoplasm to the nuclear matrix, where PML sumoylation is believed to occur (Muller et al., 1998; Zhu et al., 1997). However, rendering PML refractory to ERK1/2 phosphorylation did not affect the PML transfer to nuclear matrix induced by As_2O_3 (F.H. and M.L.P., unpublished data). Another possibility is that phosphorylation might be required for the interaction of PML with a SUMO E3 ligase. Although the combined actions of an E1-activating enzyme (Aos1/Uba2) and an E2-conjugating enzyme (Ubc9) are sufficient for in vitro sumoylation of a number of targets (Gostissa et al., 1999; Mahajan et al., 1998; Muller et al., 1998; Rodriguez et al., 1999), involvement of an additional enzyme, analogous to the E3-ligating enzymes in ubiquitination, has been proposed for some sumoylated substrates (Seeler and Dejean, 2001); several SUMO E3-like factors have been identified (Kahyo et al., 2001; Pichler et al., 2002; Sachdev et al., 2001; Kirsh et al., 2002). Certain E3 ubiquitin ligases selectively bind to and modify phosphorylated substrates (Joazeiro and Weissman, 2000); considering the similarity between sumoylation and ubiquitination, we suggest that that the phosphorylation of PML in response to As_2O_3 may alter its interaction with a known (or unknown) E3 ligase, regulating in turn PML sumoylation.

The PML-RARα fusion protein interferes with the normal functions of both PML and of RARα, and plays a crucial role in the pathogenesis of APL (Grignani et al., 1993b; Perez et al., 1993). As_2O_3 induces both partial differentiation and apoptosis of APL-derived NB4 cells through a MEK1-MAP kinase pathway (Hong et al., 2001). The prodifferentiation properties of As_2O_3 appear to be mediated through phosphorylation and inactivation of the SMRT corepressor, and the resulting derepression of PML-RARα target genes (Hong et al., 2001). Our new results, reported here, suggest that the proapoptotic effects of As_2O_3 in APL cells may be mediated, in part, through phosphorylation of PML by MAP kinase, leading to an enhanced PML sumoylation and increased apoptotic function that overcomes the anti-apoptotic properties of PML-RARα. It should be noted, however, that other effects of As_2O_3 in APL cells have been noted, including an As_2O_3-induced degradation of PML-RARα and of PML (Zhu et al., 1997, 2001). It is also likely that there are additional phenotypic changes in APL cells that may enhance their sensitivity to arsenite-induced apoptosis independent of PML and PML-RARα. More work will be required to determine the individual contributions of these different actions of As_2O_3 on the proliferation, survival, and differentiation of APL cells.

In summary, our studies provide evidence for an ERK pathway that phosphorylates PML in response to As_2O_3 exposure, and that this increase in PML phosphorylation is associated with enhanced PML sumoylation and enhanced PML-dependent apoptosis. This work provides new insight into the regulation of PML sumoylation and establishes a novel relationship between MAP kinase signaling, PML, and As_2O_3-induced apoptosis. Considering the large number of binding partners of PML, and the key contributions of PML to the stability and function of the NBs, PML phosphorylation is likely to modulate multiple cell activities beyond apoptosis through regulation of recruitment or release of NBs components.

Experimental procedures

Antibodies, plasmids, enzymes, and cell culture

The sources of antibodies, plasmids, and kinases, and the cell culture conditions are detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/5/4/389/DC1.

In vitro kinase assays

GST-PML fusion proteins were expressed in *Escherichia coli* strain BL21 and were purified by binding to, and elution from, glutathione beads (Pharmacia). ^{35}S-labeled PML was prepared using the TNT transcription/translation system (Promega). Kinase assays were performed according to the manufacturer's instructions or as previously described (Hong and Privalsky, 2000). [γ-^{32}P]-incorporation into phosphorylated proteins was detected using a STORM Imaging System (Molecular Dynamics). Molecular weight markers analyzed in parallel were visualized by Coomassie brilliant blue staining.

Transient transfections, immunoprecipitations, and immunoblottings

Cells were transiently transfected using Effectene (QIAGEN) according to the manufacturer's instructions and analyzed as previously described (Hong and Privalsky, 2000). Immunoreactive proteins were detected with the ECL-plus Western Blotting Detection System (Amersham Biosciences) using a

Fluorchem 8900 Imaging System (Alpha Innotech) or Hyperfilm (Amersham Biosciences).

Immunofluoresence

Cells (1×10^4) were seeded in a chambered coverslip cell culture system (Nalge-Nunc) and were fixed using 1:1 methanol/acetone for 10 min at −20°C. After blocking in phosphate-buffered saline containing 1% BSA, the fixed cells were incubated with anti-PML antibody (5E10) followed by incubation with FITC-conjugated secondary antibody and DAPI. Images were acquired using Microphoto EPI-FI (Nikon).

In vivo phosphorylation assays

CHO cells (5.0×10^4) were transiently transfected with appropriate PML expression plasmids (0.25 μg each) and were mock-treated, treated with 10 μM arsenic trioxide (Sigma), with 10 μM U0126 (Calbiochem), or with both for 12 hr. Total plasmid in each transfection was normalized by addition of appropriate empty vectors. PML protein in the lysates from these cells was immunoprecipitated with anti-myc antibody. Immunoprecipitates were subjected to dephosphorylation, where indicated, using calf intestinal alkaline phosphatase (Hong and Privalsky, 2000). Samples were then resolved by SDS- 8% PAGE and immunoblotting with anti-PML antibody.

In vivo sumoylation assays

CHO cells (2.5×10^4) were transiently transfected with various expression plasmids (0.125 μg each) and were mock-treated, treated with 10 μM As_2O_3, with 10 μM U0126, or with both for 12 hr. Total plasmid for each transfection was normalized by the addition of empty vectors. Cells were then lysed, and sumoylated versus unsumoylated proteins were resolved by SDS-12% PAGE and by immunoblotting with anti-myc antibody.

Cell death and apoptosis analysis

CHO cells (2.5×10^4) were transiently transfected with appropriate expression plasmids (0.06 μg each) or stably transfected with PML. NB4 cells were treated with arsenic trioxide at the indicated concentrations, with 10 μM U0126, or with both for 24 hr. Total plasmid for each transfection was normalized by the addition of appropriate empty vectors. Cell death was determined by a trypan blue exclusion assay and cell viability was calculated. Apoptotic cells were scored by TUNEL assay with the In Situ Cell Death Detection Kit (Roche Diagnostics).

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