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Promyelocytic Leukemia Is a Direct Inhibitor of SAPK2/p38 Mitogen-activated Protein Kinase*

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The promyelocytic leukemia gene (*PML*) encodes a growth/tumor suppressor protein that is essential for the induction of apoptosis in response to various apoptotic signals. The mechanism by which PML plays a role in the regulation of cell death is still unknown. In the current study, we demonstrate that PML negatively regulated the SAPK2/p38 signaling pathway by sequestering p38 from its upstream kinases, MKK3, MKK4, and MKK6, whereas PML did not affect the SAPK1/c-Jun NH_2-terminal kinase pathway. PML associated with p38 both *in vitro* and *in vivo* and the carboxyl terminus of PML mediated the interaction. In contrast to other studies of PML and PML-nuclear bodies (NB), our study shows that the formation of PML-NBs was not required for PML to suppress p38 activity because PML was still able to bind and inhibit p38 activity under the conditions in which PML-NBs were disrupted. In addition, we show that the promotion of Fas-induced cell death by PML correlated with the extent of p38 inhibition by PML, suggesting that PML might regulate apoptosis through manipulating SAPK2/p38 pathways. Our findings define a novel function of PML as a negative regulator of p38 kinase and provide further understanding on the mechanism of how PML induces multiple pathways of apoptosis.

In nearly all cases of acute promyelocytic leukemia, the promyelocytic leukemia gene (*PML*)[1] is involved in *t*(15;17) chromosomal translocation with the retinoic acid receptor α gene (1–3). The PML-retinoic acid receptor α fusion protein, encoded as a result of translocation, is thought to function as a dominant-negative factor of normal PML (4–7). PML is associated with tumor suppression, transcriptional regulation, and genomic stability (8–10). PML, a phosphoprotein associated with the nuclear matrix, is localized in the discrete, punctate nuclear structures known as PML nuclear bodies (PML-NBs), or PML oncogenic domains (11, 12). These PML nuclear bodies are critical for PML to function and are disrupted in acute promyelocytic leukemia cells. PML-NBs function as the nuclear depots (13) and they recruit many cellular components, for example, Sp100 (14), Daxx (15), p53 (16), BLM (8), and cAMP-response element-binding protein (17).

Evidence that PML plays a key role in multiple stress- and DNA damage-induced apoptotic pathways is accumulating. PML-negative cells are resistant to apoptotic stimuli, including γ-irradiation, Fas, tumor necrosis factor α (TNF-α), ceramide, and interferon (18). Consistent with this notion, the expression or distribution of PML is subject to regulation by various stimuli, such as interferon (19, 20), viral infection (21–23), heat shock (14), γ-irradiation (24), and ultraviolet (UV) irradiation (25). However, the molecular mechanisms by which PML exerts its proapoptotic activity remain to be investigated.

Extracellular environmental stimuli are transmitted to the nucleus through mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways and stress-activated protein kinase (SAPK) pathways (26–28). The MAPK/ERK pathway consists of MAPK/ERK and upstream kinases, such as ERK kinase 1 (MEK1) and Raf-1 (26, 29). The two major subfamilies of SAPKs include the SAPK1/c-Jun NH_2-terminal kinase (JNK) pathway, which consists of JNK and upstream kinases such as MAPK kinase 4 (MKK4) and MKK7; and the SAPK2/p38 pathway, which consists of p38 and upstream kinases, such as MKK3, MKK4, and MKK6 (26, 30). The JNK pathway can be stimulated by various stresses, including genotoxic stresses, and it can then activate and phosphorylate c-Jun or other transcription factors (31, 32). Similar to the activation of JNK, the activation of p38 can be mediated by various stresses, including UV irradiation, osmotic shock, TNF-α, Fas, and proinflammatory stimuli, and it can then activate and phosphorylate downstream kinases or transcription factors such as ATF2 (27, 30, 33). In this report, we investigated whether the proapoptotic activities of PML involve the SAPK signaling pathways. We show that PML associates with p38 at the endogenous levels and that PML suppresses the activity of the p38 kinase. Our data also suggest that the inhibitory effect of PML on the SAPK2/p38 pathway may account for the mechanism by which PML enhances Fas-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies (Abs) were used: mouse monoclonal anti-FLAG antibody (Sigma); mouse monoclonal anti-hemagglu-

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[1] The abbreviations used are: PML, promyelocytic leukemia gene; JNK, c-Jun NH_2-terminal kinase; PML-NB, promyelocytic leukemia gene nuclear body; Ab, antibody; KA, kinase assay; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; GST, glutathione *S*-transferase; HEK, human embryonic kidney; PI, propidium iodide; GFP, green fluorescent protein; ASK1, apoptosis signal-regulating kinase 1; PML-C, polyclonal anti-promyelocytic leukemia.

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tinin (HA) Ab (12CA5, Roche Applied Science); rabbit polyclonal anti-HA, rabbit polyclonal anti-glutathione *S*-transferase (GST), goat polyclonal anti-p38 and mouse monoclonal anti-PML (PG-M3) Abs (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-Myc and rabbit polyclonal anti-p38 Ab (Cell Signaling Technology, Inc.); and mouse anti-Fas Ab (CH-11, Upstate Biotechnology, Inc., Lake Placid, NY). Mouse monoclonal anti-PML Ab (5E10) (34) was a kind gift from Roel van Driel (Universiteit van Amsterdam, The Netherlands). Rabbit polyclonal anti-PML (PML-C) and mouse monoclonal anti-IE1 Abs were raised as previously described (22).

Mammalian Expression Plasmids and Reagents—The following plasmids were generously provided: pCMX-PML (a 560-amino acid, PML VI isoform) (3, 35) from Ronald M. Evans (Salk Institute, San Diego, CA); pcDNA3.1-Myc-ASK1 from H. Ichijo (University of Tokyo, Japan); pcDNA3-HA-MKK3 from J. Woodgett (Ontario Cancer Institute, Toronto, Canada); pEBG-GST-MKK4 from L. I. Zou (Harvard Medical School, Boston, MA); pcDNA3-HA-JNK and pcDNA3-HA-MKK6 from R. J. Davis (University of Massachusetts Medical School, MA); and pcDNA3-FLAG-p38 and pcDNA3-HA-p38 from R. J. Ulevitch (The Scripps Research Institute, La Jolla, CA). pJHA287 (1–267, PML-ΔC), pJHA289 (224–560, PML-ΔN), pJHA291 (C88P89 → S88R89 and Δ281–304, PML-ΔM), pGH623-5 (C88P89 → S88R89, PML-CP > SR), and pRL74-IE1 were previously described (22). The pGEX-4T-PML expression plasmid, which expresses GST-tagged PML (560 amino acids) in bacteria, was constructed by subcloning the EcoRI-flanked PML fragment of pCMX-PML into the EcoRI cloning site of pGEX-4T (Amersham Biosciences). SB203580 (Calbiochem) and 4′,6′-diamidino-2-phenylindole (Sigma) were purchased.

Transfections and Stable Cell Lines—Human embryonic kidney (HEK) 293 and HeLa cells were cultured as an adherent monolayer in a Dulbecco's modified Eagle's medium (Invitrogen) that was supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were transfected either with the GeneSHUTTLE-40 transfection reagent (Qbiogene, Carlsbad, CA) or by the calcium phosphate method. For the establishment of two stable cell lines, 293-Neo and 293-PML, parental HEK293 cells were transfected with the pcDNA3 plasmid (Invitrogen) or pcDNA3 plus pCMX-PML. Stable clones were selected by adding G418 (500 μg/ml). Single cell colonies were screened by immunofluorescence and immunoblot analysis with the anti-PML antibody (5E10).

Immunocomplex Kinase Assay—Cells stimulated by UV irradiation (90 J/m^2) or anti-Fas Ab (2 μg/ml) were lysed in buffer A, which contained 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 12 mM glycerophosphate, 0.1 mM orthovanadate, 10 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EGTA. Lysates were immunoprecipitated with appropriate antibodies, and the resulting immunoprecipitates were washed three times with buffer A and two times with 20 mM HEPES at pH 7.4. The immunoprecipitates were dissolved in buffer B (0.2 mM sodium orthovanadate, 10 mM $MgCl_2$, 2 μCi of [γ-^{32}P]ATP, and 20 mM HEPES at pH 7.4) with 2 μg of the appropriate substrates at 30 °C for 30 min. The following GST-fused proteins were used as substrates: GST-ATF2-(1–109) for p38; GST-c-Jun-(1–79) for JNK; and GST-p38 for MKK3, MKK4, and MKK6. The γ-^{32}P-labeled proteins were separated by SDS-PAGE and quantified with the Fuji BAS 2500 Phosphorimager.

Immunofluorescence and Confocal Microscopy—Transfected cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 followed by incubation with appropriate primary antibodies for 1 h. Bound antibodies were labeled with fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h and then visualized by MRC-1024 (Bio-Rad) confocal microscopy.

Coimmunoprecipitation and GST Pull-down Assays—For coimmunoprecipitation, cells were lysed in binding buffer C (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) and then centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatants were precleared by the incubation with protein G-Sepharose. After the added beads with nonspecific binding proteins were removed, the lysates were immunoprecipitated with the appropriate antibodies and protein G-Sepharose. The immunoprecipitates were separated by SDS-PAGE, analyzed by immunoblot assay, and visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce). For pull-down assays, [^{35}S]methionine-labeled PML was generated by using the *in vitro* transcription and translation system (Promega, Madison, WI). GST and GST-fused p38 or JNK, immobilized by glutathione-Sepharose beads (Amersham Biosciences), were incubated with *in vitro* translated [^{35}S]methionine-labeled polypeptides in binding buffer D (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 0.5% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) for 2 h at 4 °C. The washed bead-bound ^{35}S-labeled proteins were separated by SDS-PAGE and stained with Coomassie Blue or visualized by autoradiography.

Cell Death Assay—The intact cells were treated with the specific p38 inhibitor SB203580 in the appropriate concentration for 20 min and were exposed by Fas-activating anti-Fas Ab (CH-11, 100 ng/ml). After 16 h, the cells were harvested and analyzed by propidium iodide (PI) exclusion followed by flow cytometry (FACScan, BD Biosciences). In the transient transfection system, green fluorescence protein (GFP)-expressing vectors were cotransfected with target-encoding vectors for the recognition of transfected cells.

RESULTS

PML Suppresses the SAPK2/p38 Signaling Pathway—Cellular responses to a wide diversity of stimuli are mediated by MAPK and SAPK signaling pathways. The expression and function of PML are subject to the control of various stimuli but the involvement of PML in the SAPK signaling pathway remains unknown. To test whether PML affects SAPK signaling pathways, we cotransfected PML and either JNK or p38 into HEK293 cells. JNK and p38 were stimulated by UV irradiation (90 J/m^2), and kinase activity was measured by *in vitro* immunocomplex kinase assays. Interestingly, the kinase activity of p38 was reduced by 3-fold in cells transfected with PML (Fig. 1*A*), whereas the expression of PML did not significantly influence the activity of JNK (Fig. 1*B*). The reduction of p38 activity by PML is not because of a decrease in the level of the expressed p38, because comparable amounts of p38 were expressed in each transfectant as shown by immunoblotting (Fig. 1*A*, *bottom*). Similar results were obtained in the experiments where p38 was stimulated by treatment of Fas-activating Ab (α-Fas) (Fig. 1*C*) or by the transient transfection of apoptosis signal-regulating kinase 1 (ASK1) (Fig. 1*D*). An overexpression of PML blocked both the Fas- and ASK1-induced p38 activation. These results suggest that PML might be broadly involved in the SAPK2/p38 signaling pathway in response to various stresses.

To confirm these results, we used HEK293 cells that were stably expressing PML (293-PML) in a similar analysis. Immunostaining with the anti-PML antibody (5E10) displayed distinct PML-NBs in 293-PML (Fig. 2*A*), confirming an expression of significant levels of ectopic PML. A weak staining pattern, at the level of just above the background, was observed in mock-transfected cells (293-Neo), indicating that only a low level of endogenous PML is expressed in HEK293 cells. The *in vitro* kinase assay with the anti-p38 antibody showed that the kinase activity of endogenous p38 decreased by 4-fold in 293-PML as compared with the 293-Neo cells (Fig. 2*B*). Immunoblots showed that each lane contained comparable amounts of p38 protein (Fig. 2*B*, *bottom panel*). These results, consistent with the data from the transient expression system, confirm that PML indeed suppresses p38 activity.

To determine whether PML affects the kinase activity of p38 by acting on the upstream kinases in the signaling pathway cascade, we looked into the effect of PML on the p38 upstream kinases, MKK3, MKK4, and MKK6. We cotransfected HEK293 cells with cDNAs that were encoding each upstream kinase, with or without PML, and analyzed the kinase activity after UV stimulation. All three upstream kinases were activated by UV irradiation, but their activities were not affected by ectopic PML (Fig. 3). These results indicate that PML inhibits p38 activity in a manner independent of the upstream kinases and that PML might directly act on p38.

PML Specifically Interacts with p38 and the COOH Terminus of PML Is Involved in the Interaction—To further understand the mechanism underlying the negative regulation of p38

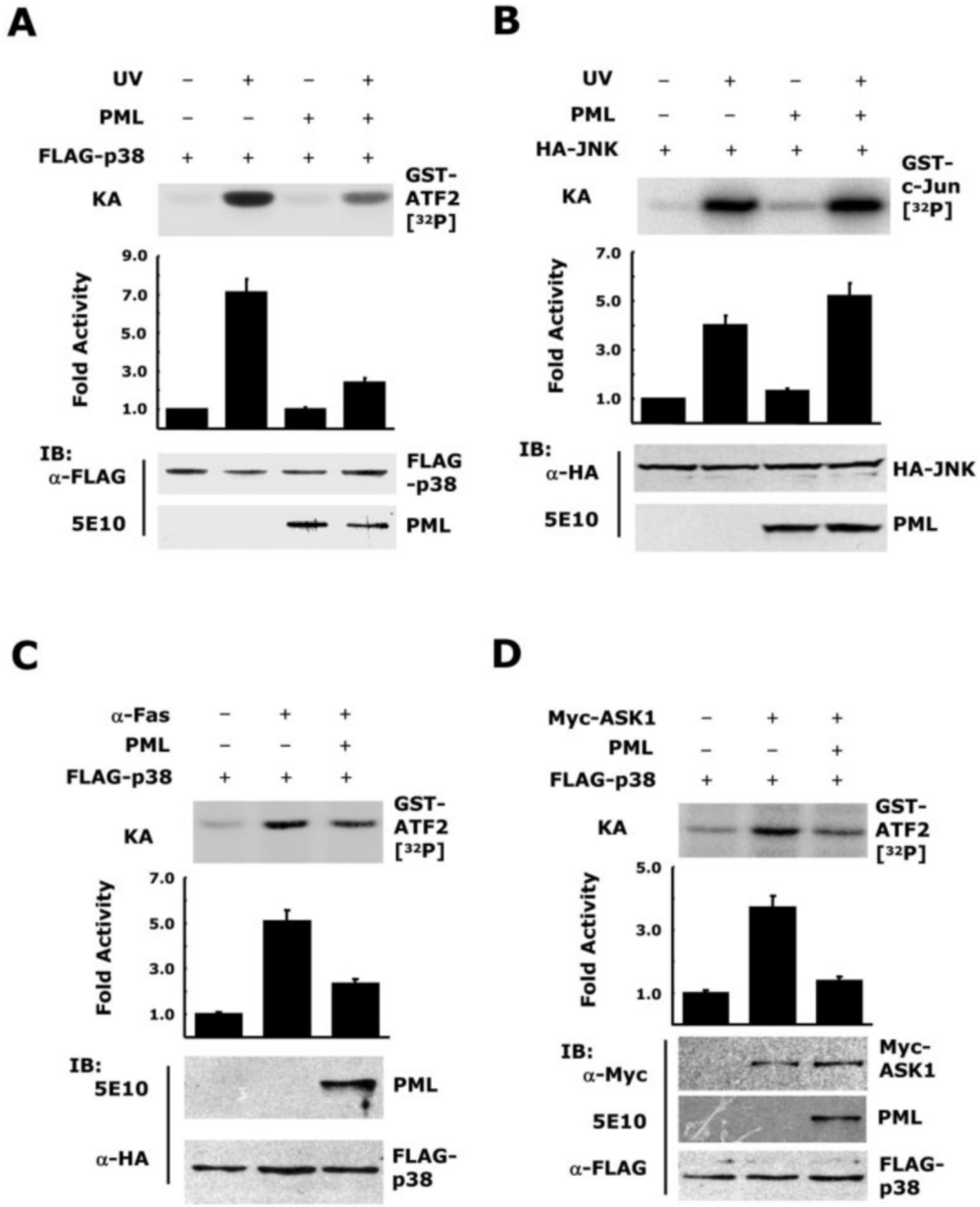

FIG. 1. **PML suppresses the SAPK2/p38 pathway.** HEK293 cells were transiently cotransfected with the combination of FLAG-p38, HA-JNK, PML, and Myc-ASK1 as indicated. For activation of p38, the cells were stimulated by exposure to UV irradiation (90 J/m^2) followed by incubation for a further 1 h (*A* and *B*) or were treated with Fas-activating anti-Fas Ab (CH-11, 2 μg/ml) for 20 min (*C*). The kinase activities of lysates were determined by an immunocomplex KA. Band intensities obtained from three independent experiments were quantitated and shown as error *bar graphs* (mean ± S.D.). *IP* and *IB* denote immunoprecipitation and immunoblot, respectively.

kinase activity by PML, we examined whether PML could physically interact with p38. We observed that only PG-M3 but not the 5E10 anti-PML antibody was able to immunoprecipitate PML (data not shown). The PG-M3 anti-PML antibody coimmunoprecipitated the p38 protein (Fig. 4*A*), demonstrating that the two proteins physically associate *in vivo*. In a reverse experiment, however, the anti-p38 antibody was unable to coimmunoprecipitate PML, suggesting that the anti-p38 antibody might disrupt or not recognize the complex.

To examine the PML-p38 interaction further, we prepared the [^{35}S]methionine-labeled PML protein by *in vitro* transcription and translation and then mixed labeled PML with GST, recombinant GST-fused p38, or JNK protein (Fig. 4*B*, *bottom panels*). A distinct ^{35}S-labeled PML protein band was retained with GST-p38 but was not present for both the control GST and GST-JNK in the GST pull-down analysis (Fig. 4*B*, *upper panel*). Although we cannot formally rule out the possibility that the interaction might be mediated through unknown molecules in transcription and translation lysates, the result reflects a specific interaction of PML with p38. Next, to elucidate the binding region of PML with p38, we used three truncated mutants lacking a COOH-terminal (1–267, PML-ΔC), NH_2-terminal (224–560, PML-ΔN), or middle region (Δ282–303 in the coiled-coil region and C88P89 > S88R89 in the RING finger, PML-ΔM) of PML. The [^{35}S]methionine-labeled PML mutants were incubated with GST-p38-conjugated beads. The PML-ΔC mutant was not bound to GST-fused p38 (Fig. 4*C*), indicating that the COOH-terminal region of PML is critical for binding p38. These results suggest that PML might exert its negative regulation of p38 kinase activity by a direct interaction with p38.

PML Inhibits Phosphorylation of p38 Mediated by MKK4 and MKK6—Because our findings thus far suggested that PML suppresses the activation of p38 through direct binding to p38, we further investigated whether PML inhibition of the p38

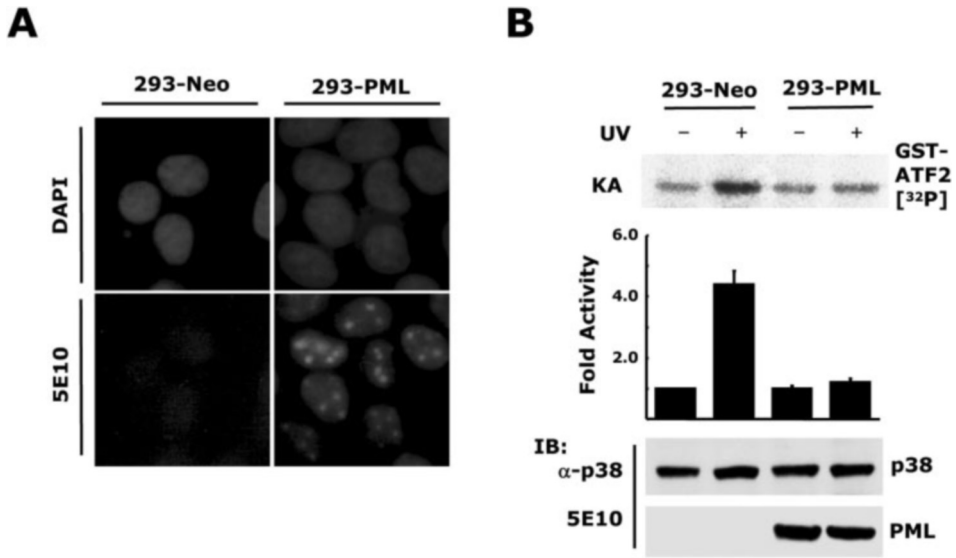

FIG. 2. **Activity of p38 is suppressed in PML stable cells.** *A*, establishment of HEK293 cell lines constitutively overexpressing PML. Neomycin-resistant cells were immunostained with mouse monoclonal anti-PML (5E10) and fluorescein isothiocyanate-conjugated anti-mouse antibodies. Cell nuclei were visualized by 4′,6′-diamidino-2-phenylindole (*DAPI*) staining. *B*, activity of the endogenous p38 in mock 293-Neo and stable 293-PML cells. These cells were exposed to UV irradiation (90 J/m^2) and lysed. The lysates were subject to immunoprecipitation using anti-p38 Ab and the resulting pellets were used for KA with GST-ATF2 as a substrate.

phosphorylation is mediated by its upstream kinases, such as MKK6 or MKK4. We expressed HA-MKK6 or the GST-MKK4 fusion proteins in the HEK293 cells and induced activation by UV irradiation. After isolating active MKK6 and MKK4 immunoprecipitates with anti-HA or anti-GST Abs, we used the immunoprecipitates for an *in vitro* immunocomplex kinase assay with GST-p38 as the substrate. In the absence of GST-PML, activated MKK6 showed robust phosphorylation of p38 (Fig. 5*A*, *top panel*, *second lane*). However, in the presence of GST-PML, the extent of p38 phosphorylation was reduced, in a dose-dependent manner, to the amount of GST-PML. Likewise, the ability of MKK4 to phosphorylate p38 was also reduced in an inverse proportion to the dose of GST-PML (Fig. 5*B*, *top panel*). The possibility that the GST portion could contribute to the inhibition of kinase activity was ruled out by KA using the control GST (Fig. 5, *A* and *B*, *bottom panels*). These data demonstrate that PML interferes with the phosphorylation of p38 mediated by its upstream kinases.

Formation of PML-NBs Is Not Essential for PML to Suppress p38—The function of PML is linked with PML-NBs, where many cellular proteins are recruited. Therefore, we determined whether the organization of PML-NBs is required for the negative regulation of p38 kinase activity by PML. To address this question, we used to our advantage the facts that the human cytomegalovirus immediate-early protein IE1 disrupts PML-NBs through direct interaction with PML and that the point mutant of PML (C88P89 > S88R89, PML-CP > SR) fails to form PML-NBs because of the mutation at the RING finger domain (22, 23). In agreement with previous findings, both the IE1 and PML-CP > SR mutant disrupted the formation of PML-NBs (Fig. 6*A*, *f*, *i*, and *l*). Accordingly, we did not observe the nuclear punctate staining of p38 in the presence of IE1 or PML-CP > SR (Fig. 6*A*, *j* and *m*). More importantly, even in the absence of PML-NB formation, PML was still capable of binding p38 (Fig. 6*B*, *top panel*, compare *third* and *fourth lanes*). Moreover, despite the disorganization of PML-NBs, PML-CP > SR could suppress the p38 kinase activity as efficiently as the wild-type PML (Fig. 6*C*). We thus concluded that direct interaction is the major determinant for PML to suppress p38 kinase activity and that the formation of PML-NBs is not required for the action of PML.

PML Promotes Fas-induced Cell Death via Suppression of p38—PML down-regulated p38 activity in the Fas- and ASK1-signaling pathways (Fig. 1, *C* and *D*). This finding prompted us to investigate the physiological effect of PML on the SAPK2/p38 signaling pathway in respect to Fas-mediated cell death. To examine the function of p38 in the Fas-induced death pathway, we used the specific p38 inhibitor SB203580, which competes with ATP for the same site on p38 (36). HEK293 and HeLa cells were treated with SB203580 at the indicated concentrations (Fig. 7, *A* and *B*, respectively). After a 20-min incubation, the cells were further incubated with or without Fas-activating anti-Fas Ab for 16 h, then stained with PI and compared with the fluorescence intensity of crude cells as a negative control. The number of PI-stained cells (M1) increased in both cell lines in proportion to the dose of SB203580 (Fig. 7), which suggests that p38 plays a role in the inhibition of Fas-induced cell death. A treatment with SB203580 alone had little influence on cell death (Fig. 7, *A* and *B*, *lane 2*, respectively). To test the effect of PML on the p38 signaling pathway in respect to Fas-mediated cell death, we analyzed Fas-induced cytotoxicity in 293-Neo and 293-PML cells (Fig. 8*A*). PML promoted Fas-mediated cell death to a similar extent as the treatment of p38 inhibitor (compare *lanes 3* and *5*). The relationship between the increase in PML protein levels and the regulation of Fas-mediated cell death through the inhibition of p38 was further confirmed in HeLa cells (Fig. 8*B*). Next, we tested whether two PML mutants with incompetence for either p38 binding or PML-NBs formation could affect the cell viability under conditions of Fas activation. In HEK293 cells, the GFP-expressing vector as a marker was cotransfected with cDNA encoding PML-wild, PML-ΔC, or PML-CP > SR. After 36 h, we treated transfectants with Fas-activating Abs and SB203580 as indicated, and examined the percentage of GFP- and PI-double positive cells by flow cytometry (Fig. 8*C*). In agreement with the above data, Fas-induced cytotoxicity was augmented by SB203580 and the wild-type PML (Fig. 8*C*, *lanes 3* and *4*), and was more enhanced by SB203580 together with the wild-type PML (Fig. 8*C*, *lane 5*). Cell death was also promoted beyond the levels of the mock transfectant with SB203580 (Fig. 8*C*, compare *lanes 3* and *7*) in the presence of the PML RING finger mutant (PML-CP > SR) that disrupts PML-NBs but is

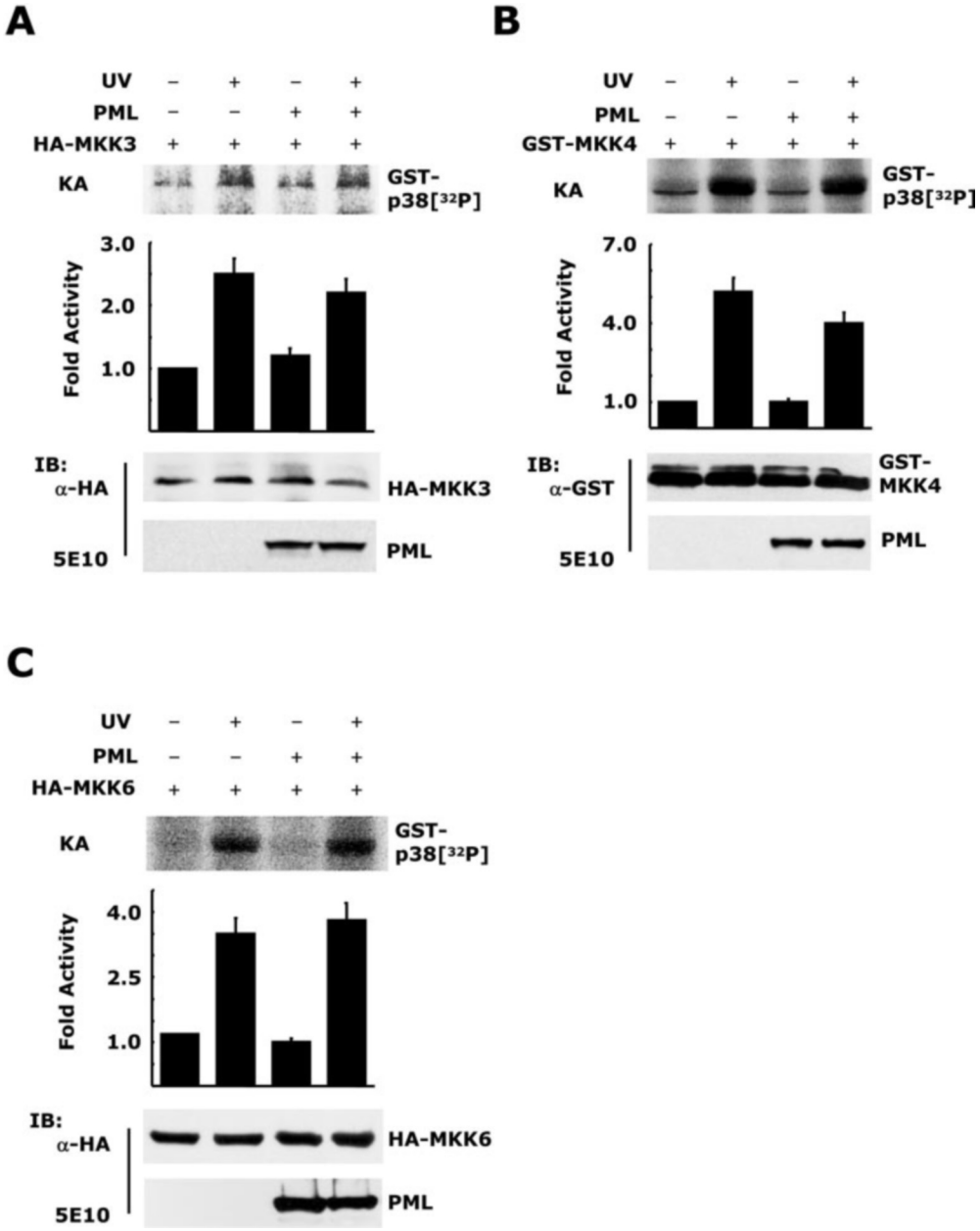

FIG. 3. **PML does not inhibit p38 upstream kinases.** HEK293 cells were transiently cotransfected with the indicated cDNAs. After 48 h of transfection, UV-stimulated cells were harvested and lysed. The lysates were analyzed for MKK3 (*A*), MKK4 (*B*), or MKK6 activity (*C*) by immunocomplex KA with GST-p38 as a substrate. Immunoblots (*IB*) confirm the expressions of the transfected plasmids.

able to suppress p38 activity (Fig. 6*C*). Interestingly, the expression of the PML-ΔC mutant, which is incapable of binding p38 (Fig. 4*C*), did not affect the Fas-mediated apoptosis (Fig. 8*C*, compare *lanes 2* and *6*). Taken together, these data suggest that PML might play a key role in promoting Fas-induced cell death via the suppression of the p38 kinase activity.

DISCUSSION

PML, initially identified as a tumor suppressor, plays an essential role in the induction of apoptosis by diverse stimuli. PML negative mice and cells are protected from these apoptotic signals (18). Furthermore, an overexpression of PML in various cell lines also induces cell death (37–39). The mechanisms by which PML exerts its proapoptotic effects remain unknown. PML is induced upon treatment with interferon, which is produced by cells in response to various biological stresses (19). Diverse biological stresses trigger the SAPKs. SAPKs are likely signaling participants in apoptosis. Therefore, the possible link between PML-induced apoptosis and the SAPK pathway has been inferred. Our data demonstrate that PML recruits p38 within the PML-NBs through physical interaction by which PML negatively regulates the SAPK2/p38 signaling pathway. In contrast, PML did not affect JNK, another SAPK family member, showing the specificity of PML to the SAPK2/p38 signaling pathway.

The p38 protein localizes to both the cytoplasm and the nucleus. It translocates between the cytosol and the nucleus in response to various stimuli (33, 40, 41) and the nucleus is a target for the signal transduction of p38 kinase (33). In agreement with previous reports, our data show that p38 is indeed localized to both the cytosol and the nucleus. Interestingly, upon overexpression of PML, the p38 protein was recruited into the PML-NBs in various cell lines including HeLa (data not shown) and HEK293 (Fig. 6*A*, *c–e*). The subcellular localization of p38 in PML-NBs has been previously implicated. Size frac-

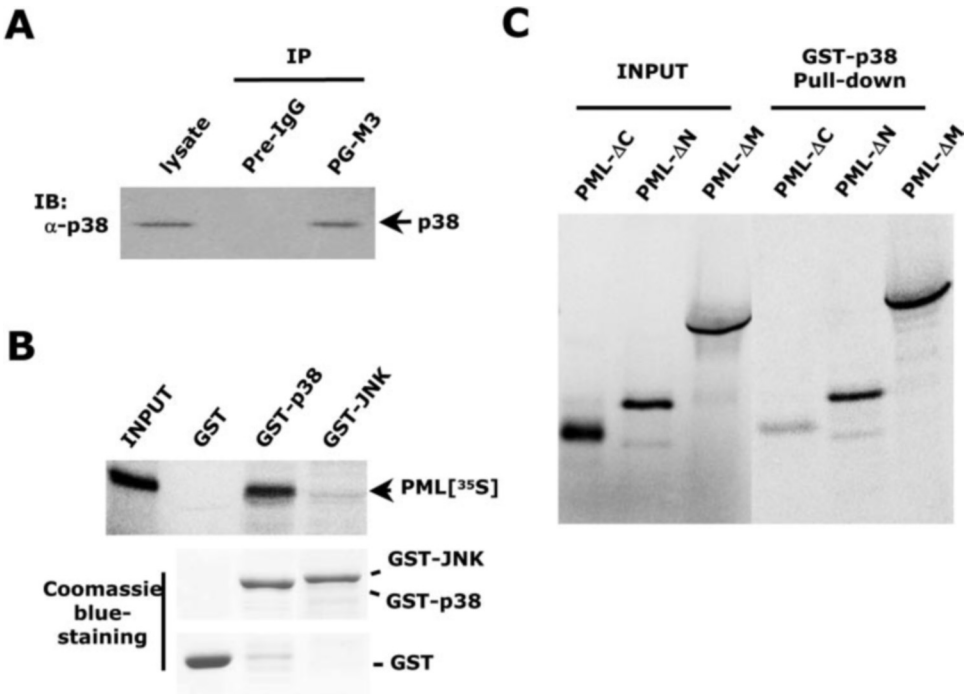

FIG. 4. **PML physically binds p38 through its COOH terminus.** *A*, HeLa cells were lysed and the *in vivo* interactions between PML and p38 were assessed by coimmunoprecipitation using the indicated Abs. Immunopellets were visualized by immunoblot analysis with the anti-p38 antibody. *B*, the *in vitro* translated [^{35}S]methionine-labeled PML polypeptides were mixed with the bacterially expressed control GST, GST-p38, and GST-JNK and immobilized onto glutathione-Sepharose beads for 2 h. The bead-bound proteins were separated by SDS-PAGE, and the ^{35}S-labeled PML proteins were visualized by autoradiography. The presence of GST and GST-fused proteins were confirmed by Coomassie Blue staining (*bottom panels*). *C*, ^{35}S-labeled PML mutant proteins were prepared and incubated with GST-fused p38 beads for 2 h. Bead-bound proteins were visualized by autoradiography. *IB*, immunoblot.

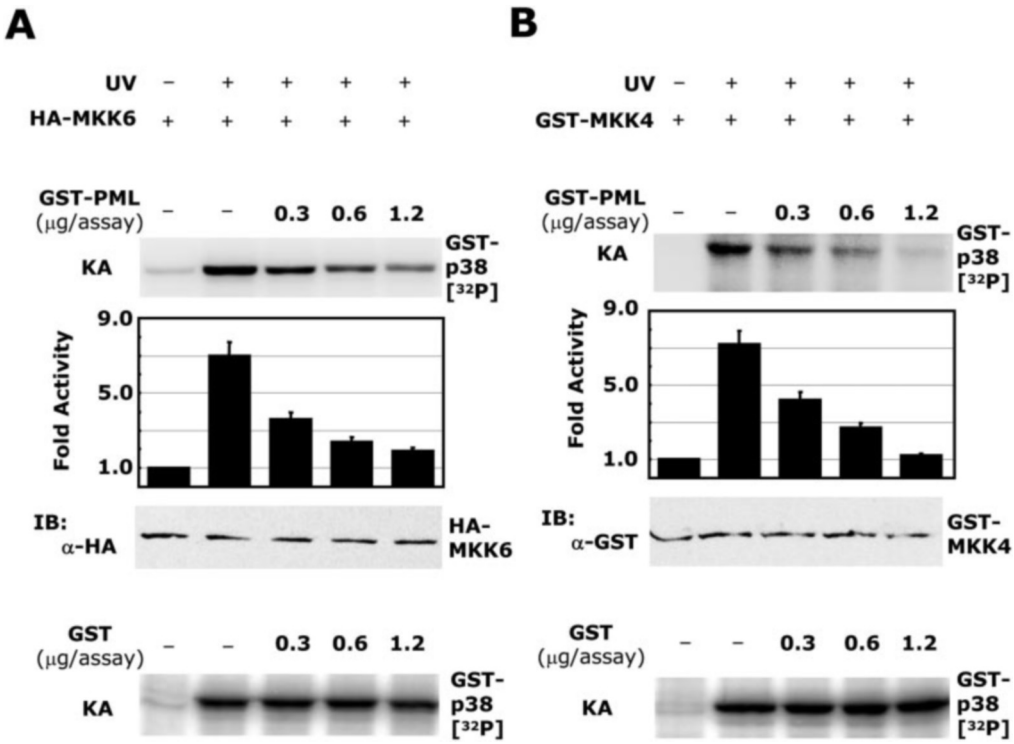

FIG. 5. **PML interferes with phosphorylation and activation of p38 catalyzed by MKK6 or MKK4.** HEK293 cells were transfected with the plasmid expressing HA-MKK6 (*A*) or GST-MKK4 (*B*). The cells were exposed to UV irradiation (90 J/m^2), incubated for a further 30 min, and then lysed. Aliquots of the lysates of UV-irradiated cells were analyzed by immunocomplex KA in the presence of variable amounts of either the recombinant GST-fused PML or the control GST epitope. The total amount of added proteins was unified by the compensation of bovine serum albumin. *IB*, immunoblot.

tionation by fast protein liquid chromatography revealed that p38 is found in 500–700-kDa subnuclear complexes that co-migrate with PML (42). Noteworthy, the p38 activator MKK3 co-migrates within these complexes. In this study, we demonstrated that PML binds p38 at the endogenous levels (Fig. 4*A*). The amount of p38 coimmunoprecipitated with PML was rela-

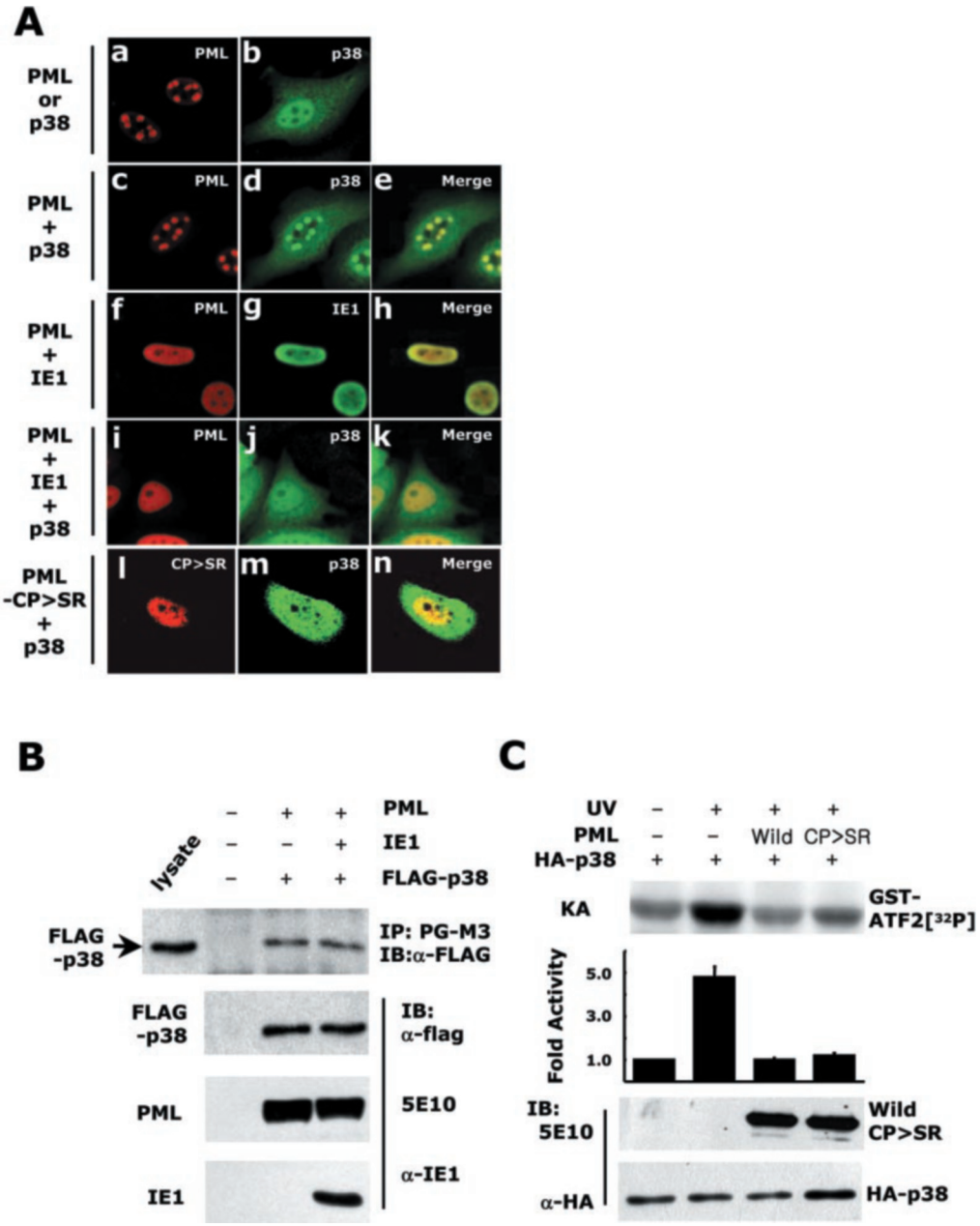

FIG. 6. **Nuclear dispersed PML is able to bind and suppress p38 activity.** HEK293 cells were transiently transfected with the combinations of plasmids as indicated. The transfected cells were analyzed by immunostaining (*A*), coimmunoprecipitation (*B*), and immunocomplex KA (*C*). *A*, cells were stained with the indicated Abs: mouse anti-HA, mouse anti-IE1, and rabbit anti-PML (PML-C) Abs. Bound primary Abs were double-labeled with Texas Red-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-mouse secondary Abs, and stained cells were visualized via MRC-1024 confocal microscopy. *B*, cell lysates were immunoprecipitated with the PG-M3 Ab, and the immunopellets were resolved by SDS-PAGE followed by immunoblotting with anti-FLAG Ab. Aliquots of cell lysates were also analyzed by immunoblot to confirm the expression of transfected plasmids (*bottom panels*). *C*, cell lysates were analyzed by immunocomplex KA.

tively small compared with the total amount of p38. Taking into account the fact that the majority of p38 is still found in the cytosol even upon the expression of PML (data not shown), the association of p38 with PML might be a nuclear event. The proteins that form complexes in PML-NBs are classified by permanent and transient groups (13), and PML-NBs are dynamic macromolecular structures in which the contents are altered in response to diverse stimuli. Several apoptotic regulators have been identified in PML-NBs, and their proper localization in PML-NBs was found to be essential for their functions (43–46). In fact, all functionally different residents of PML-NBs execute their biological functions only in the context of PML-NBs, underscoring the importance of PML-NBs. In contrast to the current understanding of PML and PML-NBs, our data show that the formation of PML-NBs is not necessary for PML to suppress p38 activity because PML could still bind and inhibit p38 activity even when the formation of PML-NBs was disrupted with either HCMV IE1 or the RING finger mutant of PML (Fig. 6). Furthermore, PML could inhibit the action of MKK4 and MKK6 on the phosphorylation of p38 *in vitro* (Fig. 5). These results support the notion that the physical binding of PML to p38 in the nucleus is a major determinant for suppressing p38 activity. The physiological importance of p38 localization in PML-NBs is not immediately clear. Given that p38 has the intrinsic ability to associate with PML, colocalization of p38 with PML within the PML-NBs might only be the consequence of a higher concentration of p38 associated with overexpressed PML, accounting for visibility in PML-NBs in

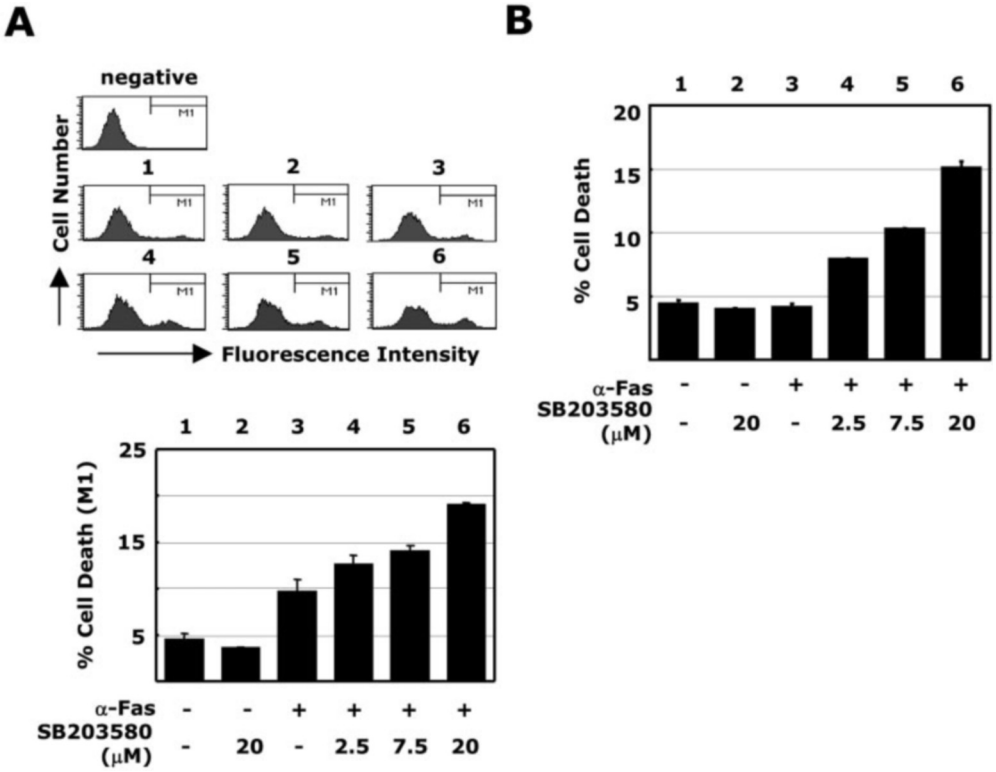

FIG. 7. **Inhibition of p38 by SB203580 promotes Fas-induced cell death.** HEK293 (*A*) and HeLa (*B*) cells were pretreated with SB203580 at the indicated dose for 20 min, incubated with anti-Fas Abs (CH-11, 100 ng/ml) for 16 h, and analyzed for cell death by PI staining using flow cytometry. The total volume of added chemicals was fixed by the addition of Me_2SO. Cells were not treated with PI in the negative control. Cell death percentages were displayed as the mean ± S.D. from three independent experiments.

comparison to visibility in the rest of the nucleus. Recruitment of a particular protein into PML-NBs does not necessarily signify the functional activation of that protein. This notion is exemplified by the fact that an increased concentration of polymerase II in PML-NBs does not increase the amount of RNA found in PML-NBs (47, 48). To date, and to the best of our knowledge, our study is the first indication that PML is able to regulate a biological function irrespective of the context of PML-NBs.

The role of the SAPK2/p38 signaling pathway in apoptosis is still controversial. The activation of p38 *in vivo* selectively induces apoptosis of CD8-positive but not CD4-positive T cells (49). The inhibition of p38 delays neutrophil apoptosis (50), whereas transient inhibition of p38 favors neutrophil apoptosis (51). The activation of p38 regulates cell survival in response to TNF-α (52). In contrast, PML potentiates cell death in TNF-α-resistant U2OS cells (53). These observations reflect the complication that the role of the SAPK2/p38 signaling pathway in regulating apoptosis could vary depending on cell type and signal quality. Because of these equivocal behaviors of p38 activities, the effect of the chemical inhibitors has been used as a hallmark for the function of p38. Diverse stimuli including UV radiation, Fas, and ASK1, activate the JNK/p38 cascade and trigger apoptosis (27, 54–56). We showed that PML negatively regulates p38 kinase activity in UV, Fas, ASK1 signalings (Fig. 1) and that the promotion of Fas-induced cell death by PML correlates with the extent of p38 inhibition by PML. The insensitivity of cells bearing resistance to Fas-induced cell death could be reversed in HEK293 and HeLa cells under conditions in which the activity of p38 is directly suppressed by SB203580 or PML (Fig. 8, *A* and *B*). Taken together, our data suggest that PML might indeed regulate apoptosis through manipulating the SAPK2/p38 pathway.

A recent study by Wu *et al.* (53) showed that the ectopic expression of PML enhances TNF-α-induced cell death by inhibiting the NF-κB survival pathway. This study demonstrated that PML IV represses the NF-κB pathway by recruiting the NF-κB subunit, RelA/p65, to the PML-NBs, and interferes with the binding of NK-κB to its enhancer. This study further describes that the COOH terminus of PML IV (amino acids 556–633) is indispensable for inhibiting NF-κB. It has also been shown that PML might be involved in p53-dependent apoptosis (57, 58). PML IV physically interacts with p53 in the PML-NBs and acts as a transcriptional co-activator with p53. Like the inhibitory action in the NF-κB survival pathway, the COOH terminus of PML IV is also required for interactions with p53 and the relocalization of p53 to the PML-NBs (57). The PML gene contains nine exons and generates many alternatively spliced transcripts, yielding a variety of PML isoforms that contain a common NH_2-terminal region but differ in their COOH-terminal sequences (35). Particular PML isoforms can mediate apoptosis by specific mechanisms (35), reflecting that PML splice variants might evolve to respond to diverse signals. Throughout our study, we used the PML VI isoform (560 amino acids) that lacks the COOH-terminal region of PML IV, and thus it is unlikely that the mechanisms by which PML accelerates Fas-mediated cell death involves the inhibition of the NF-κB survival pathway and induction of the p53-dependent apoptotic pathway. Daxx, as an another factor in PML-NBs, has been known to participate in the apoptotic pathway via direct interaction to PML VI, and its localization in PML-NBs is required for apoptotic functions (59–61). In contrast, the PML mutant that failed to form PML-NBs did still bind and suppress p38 activity (Fig. 6), and exerted the physiological effect that was lower than PML-wild but still drew level with the p38 inhibitor (Fig. 8*C*). These data give a clue that certain PML-NBs partners, including Daxx, could participate in PML-mediated apoptosis although PML might also have its own

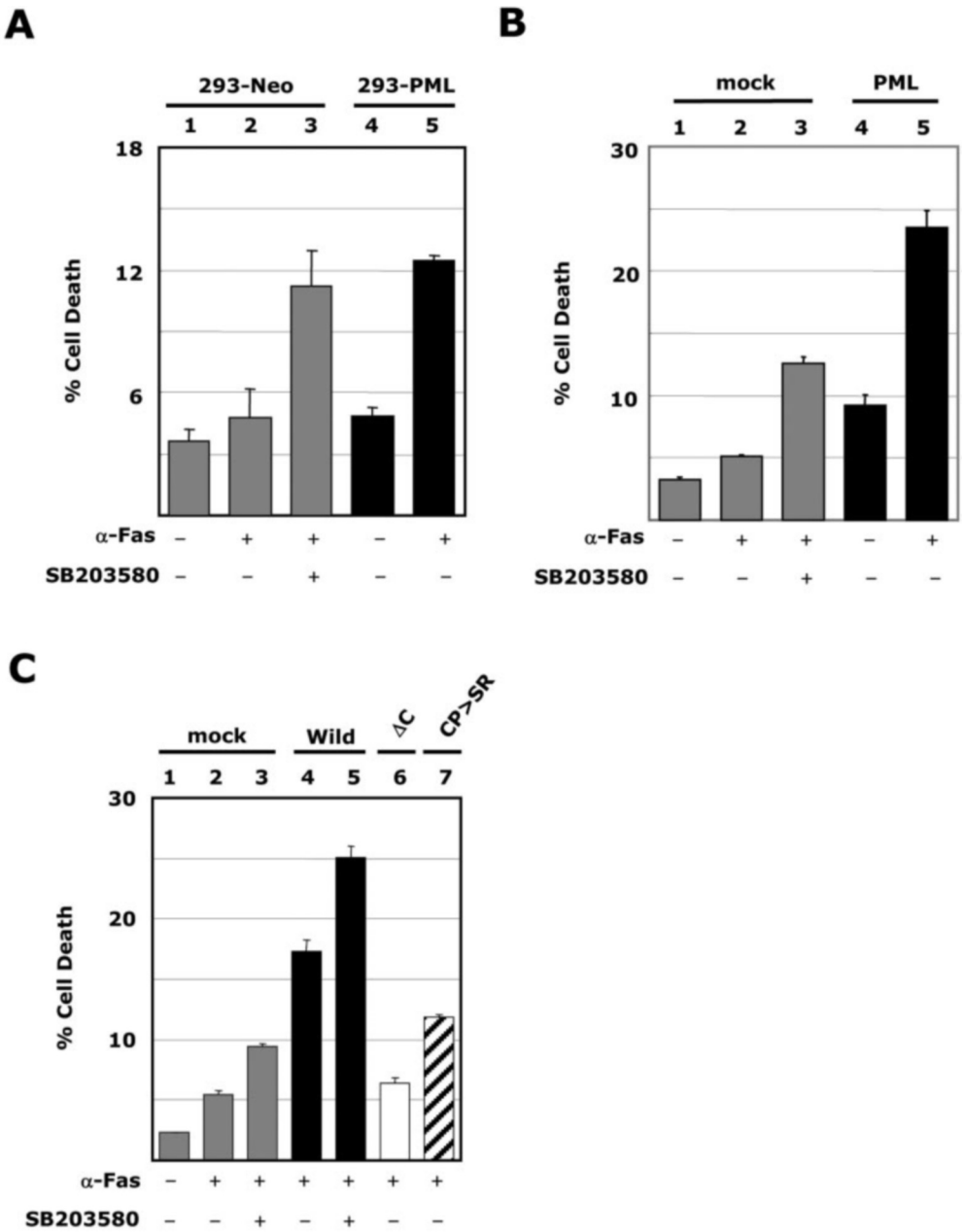

FIG. 8. **Inhibition of p38 by PML promotes Fas-induced cell death.** *A* and *B*, HeLa cells were transfected with GFP plus mock or PML-encoding vectors. 293-Neo, 293-PML (*A*), and HeLa transfectants (*B*) were treated with Fas activating anti-Fas Abs in the presence or absence of SB203580 (20 μM), and then analyzed for PI exclusion. *C*, indicated cDNA was cotransfected with GFP-vector into HEK293 cells. Transfectants were treated with anti-Fas Abs in the presence or absence of SB203580 (20 μM), and then analyzed for the GFP- and PI-double positive cells by flow cytometry. Error *bar graphs* represent the mean ± S.D. of death percentage from three independent experiments.

capability. PML exhibited synergy with SB203580 to potentiate sensitivity to Fas-mediated cell death in our experimental conditions (Fig. 8*C*), which suggests that inhibition of the SAPK2/p38 pathway could increase the apoptotic function of other factors in PML-NBs such as the inhibition of Daxx by downstream Hsp27 (62, 63). Alternatively, the synergistic action of SB203580 and PML might be a reflection that these chemical and cellular inhibitors target different steps of the SAPK2/p38 pathway, for instance, competition with ATP and sequestration from upstream kinases, respectively. Our novel finding that PML, as a natural inhibitor for p38, plays a role in stress signaling cascades, can contribute to a further understanding of the role of PML in apoptosis.

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