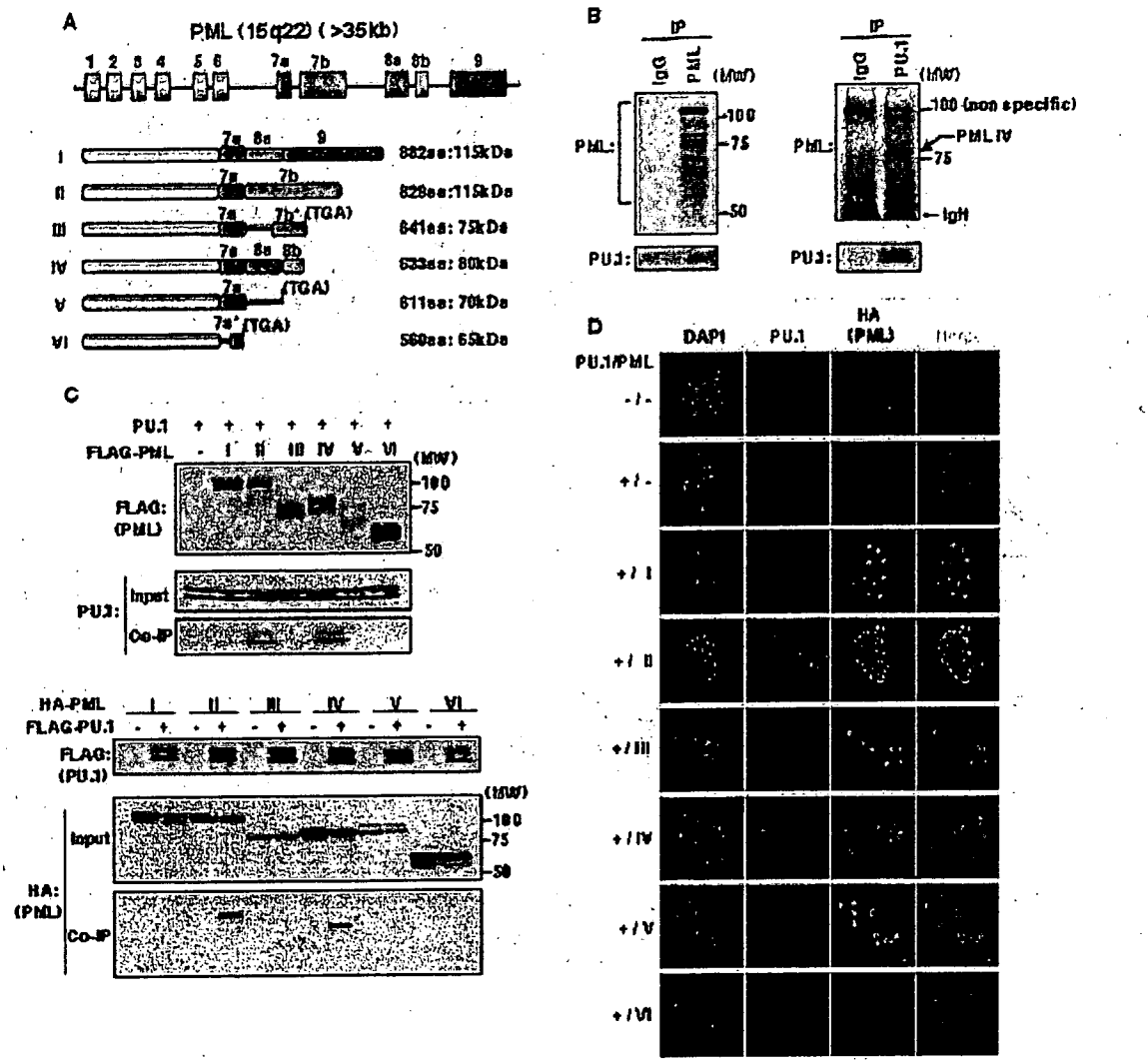


FIG. 3. PML associates with PU.1 in vivo. (A) Schematic illustration of the genomic structure of the *PML* gene. Boxes represent exons, and their exon numbers are indicated. Six major alternatively spliced isoforms are shown. The solid lines indicate retained introns. Asterisks show frameshifts of the coding sequence compared to PML II. Numbers of amino acid (aa) residues and the apparent molecular masses of each isoform are given. (B) Association of endogenous PU.1 and PML. Total cell lysates from HL-60 cells were immunoprecipitated with an anti-PML (left) or an anti-PU.1 (right) antibody and then analyzed by Western blotting. Note that PML, with a molecular mass of ~75 kDa, was coprecipitated predominantly with PU.1. IgG, immunoglobulin G. (C) PU.1 coimmunoprecipitates with PML II and IV. Total cell lysates from BOSC23 cells transfected with the indicated expression vectors were subjected to immunoprecipitation with an anti-FLAG antibody and then analyzed by Western blotting. The antibodies used for Western blotting are indicated on the left of each panel. IP, immunoprecipitates; MW, molecular weight (in thousands). (D) PU.1 and PML IV were colocalized within PODs. The expression vectors indicated were transiently coexpressed in NIH 3T3 cells and then costained with antibodies to PU.1 and HA (for PML). DAPI, 4′,6′-diamidino-2-phenylindole.

After 7 days of culture with $ZnSO_4$, most of the control mock-transfected cells (L-G/MT-PU.1/mock) differentiated around the metamyelocyte stage, and only a few mature PMNs were observed. In contrast, more than 60% of L-G/MT-PU.1/PML IV cells differentiated into mature PMNs. The PML VI isoform cooperated moderately with PU.1 to induce granulocytic differentiation (see Fig. S3D in the supplemental material). The other PML isoforms (I, II, III, and V), however, did not affect PU.1-induced differentiation of L-G cells (data not shown). It is noteworthy that the cooperativity of PU.1 and PML isoforms in granulocytic differentiation was comparable to their POD colocalization capability.

Next, we examined the effect of PML IV on PU.1 transcription activity. Luciferase reporter assays showed that among six PML isoforms, only PML IV had a marked effect on the activation of the *C/EBPε* reporter by PU.1 (Fig. 4D). A parallel experiment using a reporter of the *M-CSFR* promoter also demonstrated a specific cooperation between PU.1 and PML IV, indicating that the interaction between these two proteins does not depend on the promoter context (see Fig. S3E in the supplemental material).

Moreover, we next examined whether PML IV could affect the expression of endogenous C/EBPε during PU.1-induced granulocytic differentiation (Fig. 4E). In L-G/MT-PU.1/mock

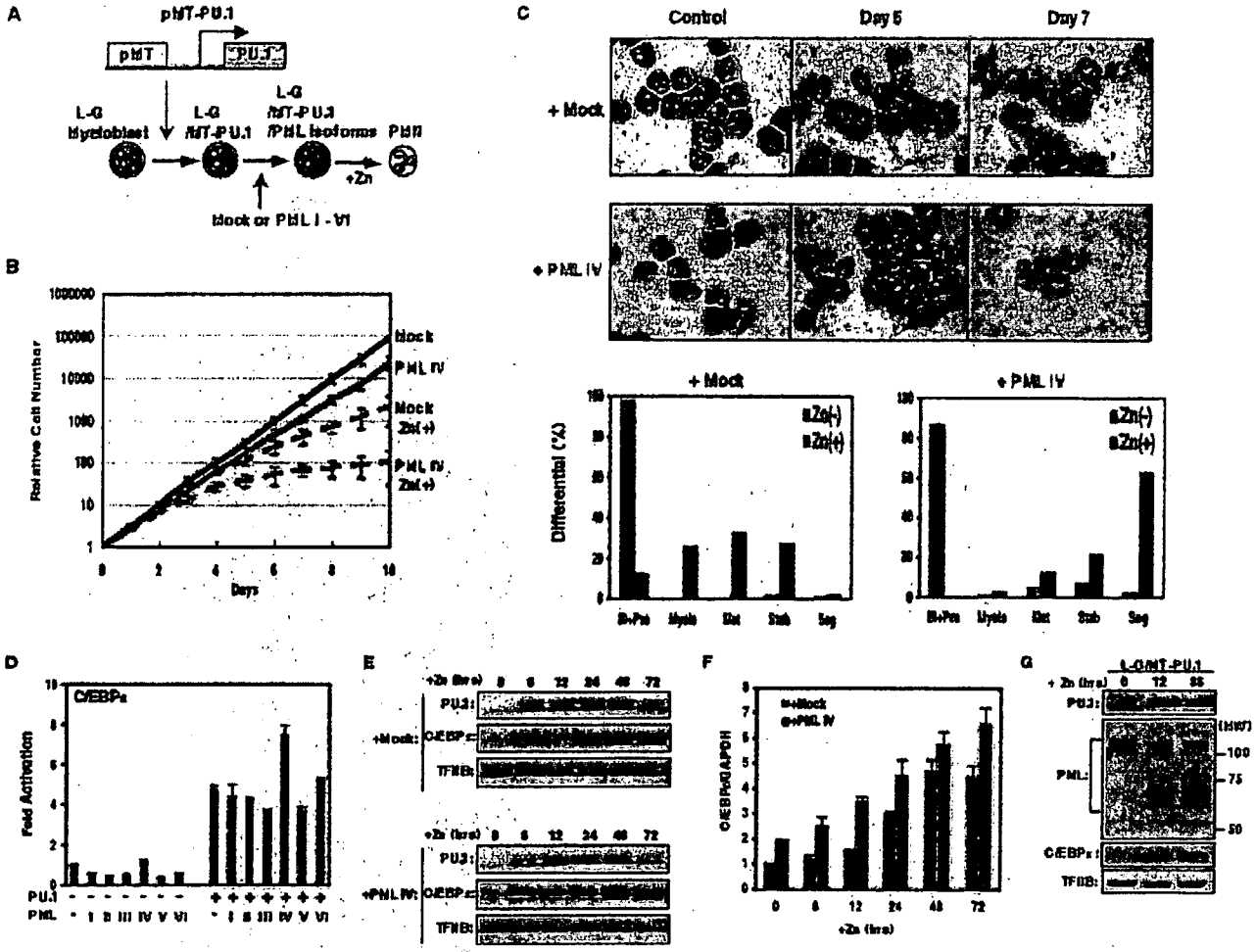


FIG. 4. PML IV and PU.1 cooperate to accelerate terminal differentiation of L-G myeloblasts. (A) Construction of L-G cells transfected with a plasmid encoding PU.1 under the control of the metallothionein promoter (pMT-PU.1). Stable clones could be induced to differentiate into PMNs. The cells were further transformed with each PML isoform or mock using a retroviral vector, generating L-G/MT-PU.1/PML I to VI or mock, respectively. (B) Upon expression of PU.1, PML IV synergistically suppressed the proliferation of L-G cells. (C) Differentiation of L-G cells upon induction of PU.1 by $ZnSO_4$ treatment. Cytospin-prepared cells were stained with May-Giemsa stain (top) and evaluated by morphological criteria after 7 days (bottom). Bl, blast; Pro, promyelocyte; My, myelocyte; Met, metamyelocyte; Stab, stab cell; Seg, PMNs. (D) PML IV specifically enhances PU.1-induced activation of the *C/EBPε* promoter-containing luciferase reporter in NIH 3T3 cells. The effector plasmids are indicated. (E) Western blotting shows that PML IV and PU.1 synergistically enhance the expression of C/EBPε in L-G cells. (F) Real-time RT-PCR was used to quantify *C/EBPε* mRNA in L-G/MT-PU.1/mock and PML IV cells treated with $ZnSO_4$ for the indicated times. All results are given in relative units compared to *GAPDH*. Result are means ± standard deviations of triplicate determinations of a representative experiment. Note that PCR detects all *C/EBPε* mRNA isoforms generated by the alternative use of promoters or splicing. (G) Western blotting shows that the expression of endogenous PML protein increases during PU.1-induced granulocytic differentiation. MW, molecular weight (in thousands).

cells, C/EBPε expression started to increase 24 h after exposure to $ZnSO_4$, and it reached a maximum after 48 to 72 h. On the other hand, the coexpression of PML IV enhanced C/EBPε expression within 6 h after $ZnSO_4$ treatment in parallel with PU.1 expression. The PML VI isoform modestly promoted PU.1-induced C/EBPε expression. The other PML isoforms (I, II, III, and V), however, did not affect PU.1-induced expression of C/EBPε (see Fig. S3F in the supplemental material).

To confirm that PML IV enhancement of C/EBPε expression was due to transcriptional activation, quantitative RT-PCR was performed (Fig. 4F). In L-G/MT-PU.1/PML IV cells, all six time points showed elevated *C/EBPε* transcripts compared to L-G/MT-PU.1/mock cells. The difference was more prominent before C/EBPε expression started to increase in L-G/MT-PU.1/mock cells.

We next investigated why more than 12 h was required before the induction of C/EBPε expression in LG/MT-PU.1 cells in spite of possible direct regulation by PU.1. Western blots showed that PU.1 induced the expression of endogenous PML in L-G/MT-PU.1 cells (Fig. 4G). PML expression did not increase after $ZnSO_4$ treatment in either parent L-G cells or L-G/MT-PU.1 cells driven to differentiate into granulocytes by treatment with granulocyte colony-stimulating factor instead of interleukin-3 (see Fig. S3G in the supplemental material). These results indicate that endogenous PML expression is specifically regulated by PU.1. Since quantitative

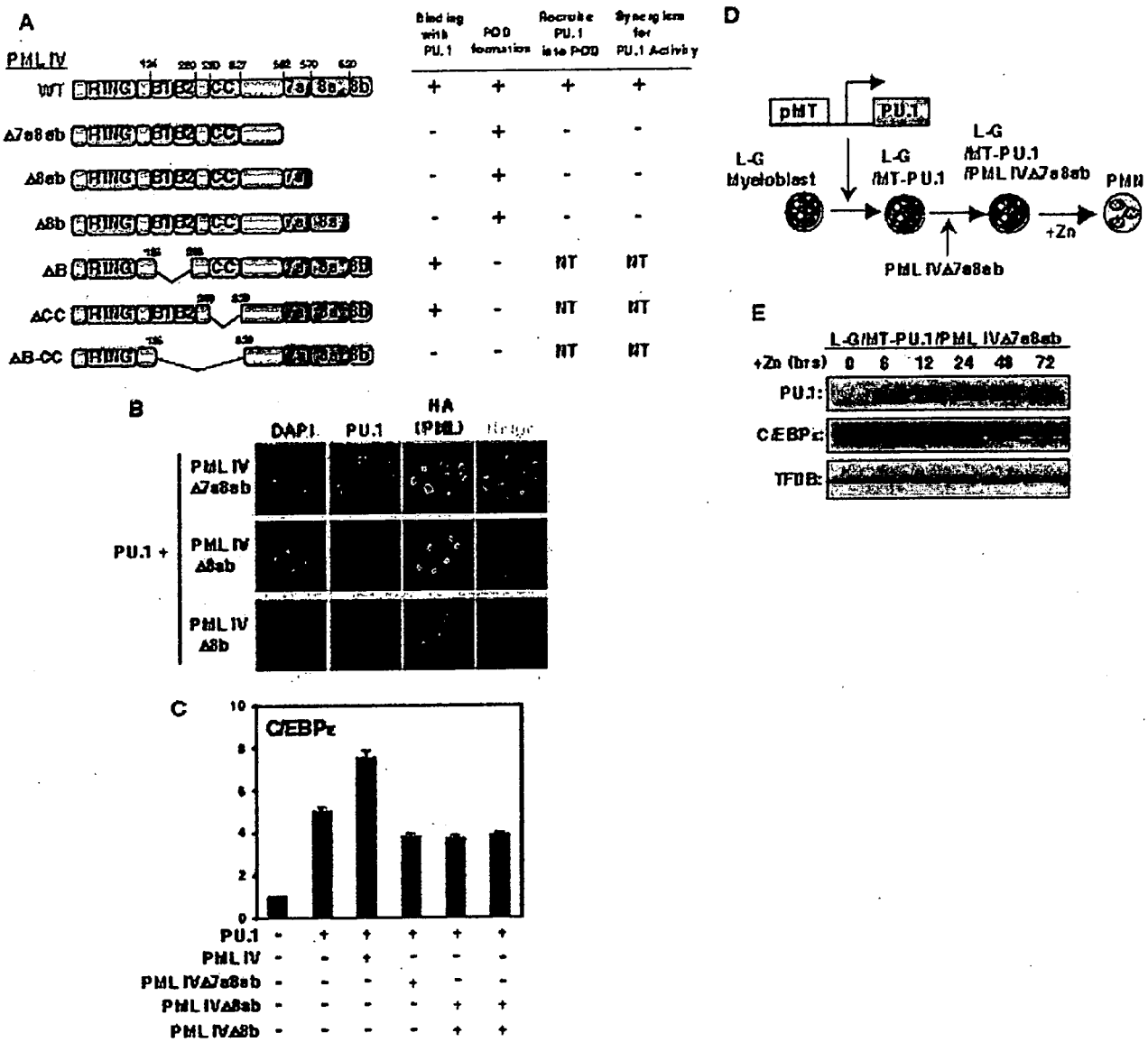


FIG. 5. Enhancement of PU.1-induced terminal differentiation of L-G cells requires the C terminus of PML IV. (A) Schematics of the PML IV mutants and summary of the domain mapping of the physical and functional interaction with PU.1. Pro, proline-rich region; RING, RING finger domain; B1 and B2, B boxes; CC, coiled-coil domain; WT, wild type; NT, not tested. (B) Immunofluorescence shows that the PML IV C-terminal deletion mutants do not colocalize with PU.1 in NIH 3T3 cells. DAPI, 4′,6′-diamidino-2-phenylindole. (C) Luciferase reporter assays show that PML IV C-terminal deletion mutants do not enhance PU.1-induced transcription in NIH 3T3 cells. (D) Schematics of the construction of PU.1-inducible L-G cells transduced with a PML IV C-terminal deletion mutant (L-G/MT-PU.1/PML IVΔ7a8ab) or mock (L-G/MT-PU.1/mock). (E) Western blots show that the PML IVΔ7a8ab is unable to enhance PU.1-induced expression of C/EBPε in L-G cells.

RT-PCR analysis revealed that endogenous *PML* mRNA also increased (data not shown), PU.1 regulates PML expression, at least in part, at the transcriptional level. An important finding is that C/EBPε expression was also induced in a fashion parallel to that of PML expression in L-G/MT-PU.1 cells (Fig. 4G). Together with the finding that the induction of C/EBPε becomes much faster when PML IV is already expressed exogenously (see Fig. S3H in the supplemental material), these results clearly indicate that PU.1 action on *C/EBPε* transcription is modulated by PML.

Structure-function relationship of the PU.1-PML IV interaction and its relevance in myeloid terminal differentiation. We performed coimmunoprecipitation assays to determine the region of PML required for the association with PU.1. Deletion of the C-terminal 13 amino acids of PML IV, which corresponds to the isoform-specific exon 8b, completely abolished the formation of the PU.1-PML complex (Fig. 5A) (see Fig. S4A in the supplemental material). The integrity of B boxes and the coiled-coil region was also required for an association with PU.1 (Fig. 5A) (see Fig. S4A in the supplemental mate-

rial). In addition, these C-terminally-deleted PML mutants could no longer recruit PU.1 to PODs in vivo (Fig. 5B), nor could they enhance PU.1-mediated transcription (Fig. 5C). Because the deletion mutant lacking exon 8b was unstable, and another one lacking exons 8a and 8b (PML IVΔ8ab) was not efficiently expressed in L-G cells (see Fig. S4B and S4C in the supplemental material), we used L-G/MT-PU.1 cells expressing PML lacking exons 7a, 8a, and 8b (PML IVΔ7a8ab) for further analysis (Fig. 5D). In addition to losing the colocalization and transcriptional cooperation with PU.1, PMLΔ7a8ab had no effect on the profile of PU.1-induced C/EBPε expression and cell differentiation (compare Fig. 5E and S4D and S4E in the supplemental material with Fig. 4B, C, and E).

We also performed reciprocal experiments employing PU.1 mutants. Coimmunoprecipitation analysis using PU.1 deletion mutants showed that the acidic amino-acid-rich region (DE region) of the PU.1 transactivation domain is necessary for an association with PML IV (Fig. 6A) (see Fig. S5A in the supplemental material). As expected, PML IV could no longer recruit PU.1 lacking the DE region (PU.1ΔDE) to PODs in vivo (Fig. 6B, top), nor could it enhance PU.1ΔDE-mediated transcription, although PU.1ΔDE itself has a weak ability to enhance transcription (Fig. 6C, lanes 5 and 6). In agreement with these results, the expression of PU.1ΔDE did not effectively induce the expression of C/EBPε, and the overexpression of PML IV could not rescue the expression of C/EBPε (Fig. 6E, top) or significantly affect the differentiation of L-G cells (see Fig. S5D and S5E in the supplemental material).

To confirm the significance of the physical interaction between PU.1 and PML IV, we performed parallel experiments using a PU.1ΔPEST mutant that retains the ability to bind PML IV (see Fig. S5A in the supplemental material). In contrast to PU.1ΔDE, PML IV enhanced PU.1ΔPEST-mediated transcription to an extent similar to that of wild-type PU.1 (Fig. 6C, lanes 7 and 8). PML IV also enhanced C/EBPε expression in L-G/MT-PU.1ΔPEST cells, although it did not affect the time course of C/EBPε expression (Fig. 6E, bottom). This cooperation between PML IV and PU.1ΔPEST was also observed in the granulocytic differentiation of L-G cells (see Fig. S5H and S5I in the supplemental material). Interestingly we noticed that PU.1ΔPEST expression in PML IV-transduced cells was maintained at a high level even after 48 h of treatment of $ZnSO_4$ compared to mock-transduced cells. RT-PCR analysis revealed that mRNA expression of PU.1ΔPEST was equal in both cells (see Fig. S5J in the supplemental material). These results suggest that PML IV enhances PU.1ΔPEST expression by a posttranscriptional mechanism.

Taken together, these results demonstrate that the specific interaction between PU.1 and PML IV is involved in their abilities to promote granulocytic differentiation.

PML IV promotes the association of PU.1 and p300 during granulocytic differentiation to form complexes for active expression of C/EBPε. We next investigated the significance of the interaction of PU.1 and PML in regulating C/EBPε expression during granulocytic differentiation. In HL-60 cells, RA treatment immediately increased the expression of PU.1, PML, and p300 for 48 h (Fig. 7A), which thereafter decreased (data not shown). The expression of all PML isoforms increased evenly. C/EBPε expression markedly increased for 48 h, whereas C/EBPβ expression transiently increased and then returned to a level equal to that of untreated cells. To determine the interaction of the ternary complex of PU.1/PML/p300 on the *C/EBPε* promoter, ChIP analysis was performed (Fig. 7B). Upon RA treatment, promoter-associated PU.1 modestly increased, and PML association gradually increased. A rapid recruitment of p300 within 24 h of RA treatment, which may be mediated by promoter-bound RAR through an RA-responsive element (RARE), was followed by further accumulation after 48 h of treatment. Note that the amount of p300 that coimmunoprecipitated with PU.1 was only minimally detected in untreated HL-60 cells but significantly increased within 48 h by RA treatment, and this increase was proportional to the amount of PML coimmunoprecipitation rather than the amount of PU.1 itself (Fig. 7C). These results demonstrate that the ternary complex of PU.1/PML/p300 forms on the *C/EBPε* promoter and that the association of the complex increases in parallel to PML recruitment on the promoter during the early stage of granulocytic differentiation.

PU.1 alone induced C/EBPε expression, although relatively slowly, in L-G cells (Fig. 4E and 7D). An interesting finding is that C/EBPε expression was induced proportionally to p300 coimmunoprecipitation with PU.1. Another important finding is that the amount of p300 coimunnoprecipitation with PU.1 increased proportionally to the expression level of PML (Fig. 7D). These results suggest a scaffold function of PML in the association of PU.1 and p300. To confirm this, the association of these proteins was further examined by immunoprecipitation experiments using a transient expression system. Although the interaction between p300 and PU.1 is seemingly enhanced by the coexpression of PML IV (Fig. 7E, top panels, lanes 3 and 4), the coexpression of PML IV also increased PU.1 expression. These results raised the possibility that the increased coimmunoprecipitation of p300 may be due simply to an increased expression and availability of PU.1. To exclude this possibility, a reciprocal experiment was performed. Coimmunoprecipitation of PU.1 with p300 was not efficiently detected in the absence of PML IV but was easily observed when PML IV was coexpressed (Fig. 7E, middle panels, lanes 3 and 4). On the other hand, the coexpression of PU.1 did not affect the interaction between p300 and PML IV (Fig. 7E, bottom panels, lanes 3 and 4). These results suggest that the association of PU.1 and p300 is more labile than that of PML and p300 and support the data obtained in HL-60 cells showing that it can be stabilized by PML IV. We next performed immunofluorescence experiments to further confirm whether PU.1, p300, and PML form ternary complexes in vivo (Fig. 7F). Whereas both PU.1 and p300 localized throughout the nucleus, they were concentrated in PODs when PML IV was coexpressed. We then investigated the cooperation of PML IV and p300 in PU.1-induced transcription by luciferase reporter assays (Fig. 7G). PU.1 activation of the *C/EBPε* promoter was only slightly enhanced by the coexpression of p300 alone, but it was synergistically enhanced by the coexpression of PML IV and p300.

Notably, PU.1 was not efficiently recruited to abnormal nuclear aggregates of a sumoylation-deficient PML IV-3R mutant (see Fig. S6A in the supplemental material), even though PML IV-3R could still associate with PU.1 in immunoprecipitation experiments (data not shown). In contrast, p300 still efficiently colocalized with PML IV-3R. In agreement with its inability to recruit PU.1, PML IV-3R did

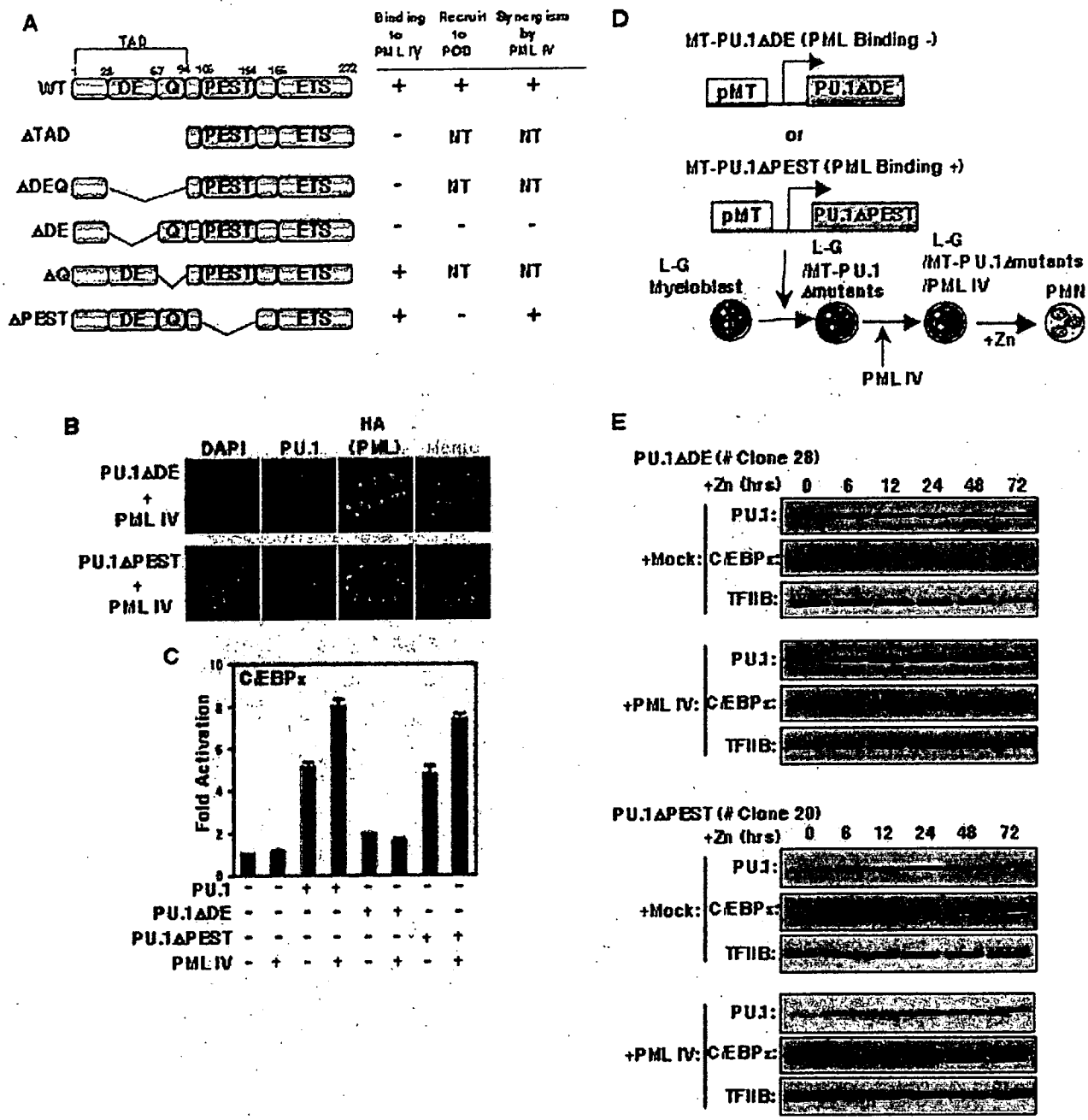
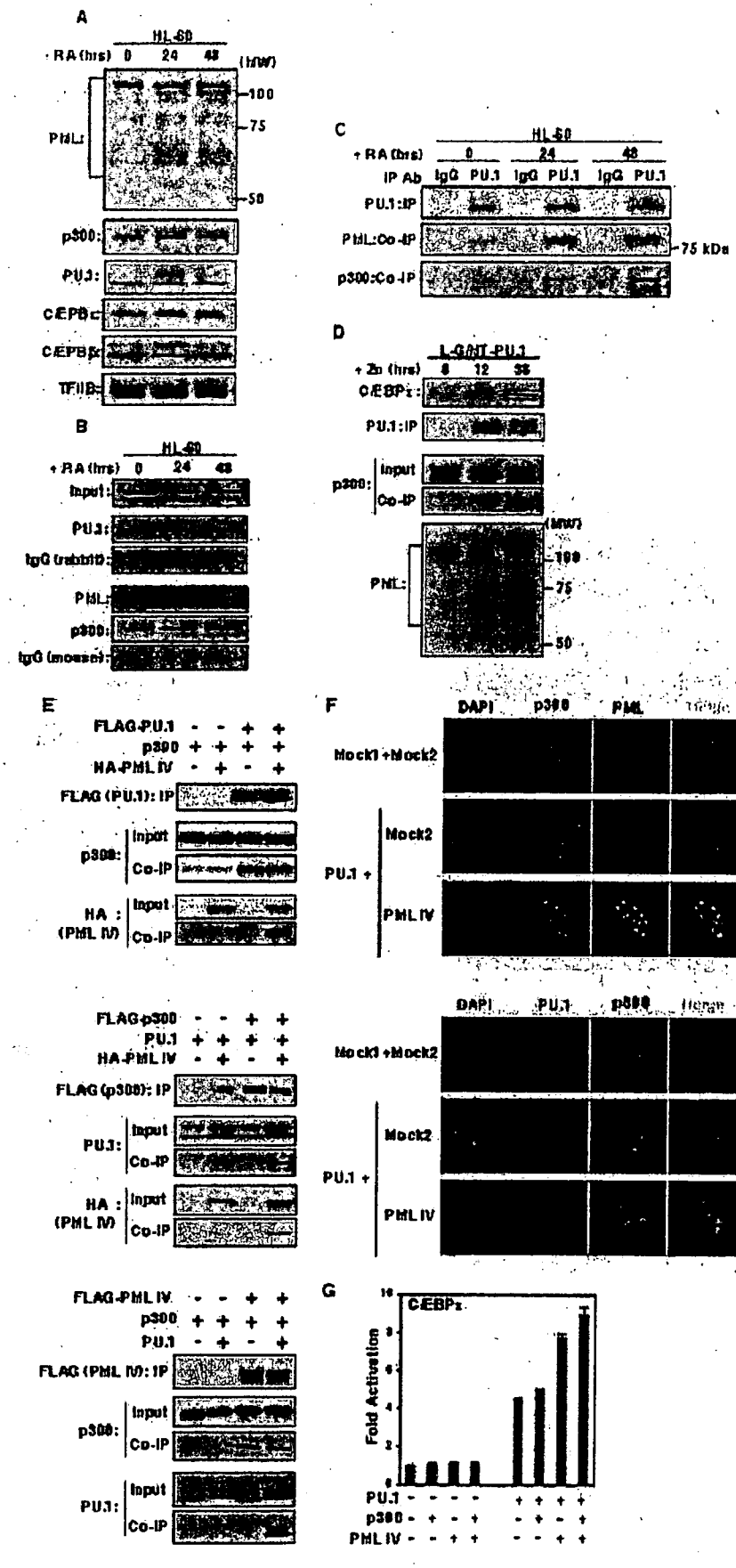


FIG. 6. The PU.1 transactivation subdomain is required for the enhancement of terminal differentiation by PML IV in L-G cells. (A) Schematics of PU.1 mutants and summary of the domain mapping of the physical and functional interaction with PML IV. TAD, transactivation domain; DE, acidic amino acid-rich region; Q, glutamine-rich region; ETS, *ets* DNA-binding domain; WT, wild type; NT, not tested. (B) Immunofluorescence shows that the PU.1 mutants do not colocalize with PML IV in NIH 3T3 cells. DAPI, 4′,6′-diamidino-2-phenylindole. (C) Luciferase reporter assays show that PML IV does not enhance the induction of transcription by the PU.1ΔDE. (D) Schematics of the L-G myeloblast clones transduced with mutant forms of PU.1. Those cells were further transduced with PML IV (L-G/MT-PU.1 Δmutants/PML IV) using a retroviral vector. (E) Western blots show that PML IV enhances the induction of C/EBPε expression by PU.1ΔPEST but not by PU.1ΔDE.

not enhance PU.1 transactivation (see Fig. S6B in the supplemental material). These results suggest that a normal POD structure would be crucial for transcriptional synergism between PU.1 and PML IV.

PML-RARA disrupts active PU.1/PML/p300 transcriptional ternary complex. We next tested whether PML-RARA can affect PU.1-induced transcription. The *C/EBPε* promoter contains RARE. We found that whereas ligand-unbound RARA



represses *C/EBPε* promoter activation, RA releases it to allow PU.1 to transactivate *C/EBPε* expression (see Fig. S7A in the supplemental material). PML-RARA also repressed promoter activity in the absence of RA (Fig. 8A). We also found that the enhancement of PU.1-induced transcription by PML IV was greatly reduced by the coexpression of a much lower amount of PML-RARA, suggesting its potent dominant-negative effect on PML IV. In addition, PML-RARA repressed the transactivation by PU.1 even in the absence of exogenous coexpression of PML IV.

To determine whether the direct recruitment of PML-RARA to the promoter is required for its inhibitory effect, we performed transactivation experiments using the RARE-mutated (*C/EBPε-mtRARE*) reporter to which PML-RARA could no longer bind. Neither PML-RARA nor the RARA/retinoid X receptor affected the reporter activity in the absence of RA (data not shown); however, PML-RARA still dose-dependently inhibited both PU.1 transactivation and the PML IV enhancement of PU.1-induced transcription, similar to the wild-type reporter (Fig. 8B). These results suggest that the inhibition of *C/EBPε* expression by PML-RARA is caused by the targeting of the PU.1-transcription factor complex. The *M-CSFR* promoter is also transrepressed by PML-RARA (data not shown), indicating that the effect of PML-RARA does not depend on the promoter context. Furthermore, PLZF-RARA, another APL-related chimera, disrupts the normal POD structure (20) and has effects that are similar to those of PML-RARA (see Fig. S7B in the supplemental material). These results suggest that the POD structure is required for PU.1 transactivation and is targeted by both chimeras.

To examine the inhibitory effects of PML-RARA on a different class of transcription factors, we performed parallel experiments using AML1b, which is functionally modulated by PML I (Fig. 8C). In contrast to its effects on PU.1, PML-RARA only partially attenuated the PML I enhancement of AML1b transcription. To exclude the possibility that the inhibitory action of PML-RARA is directed towards PML IV function, another parallel experiment was performed. c-Myb was also associated with and superactivated by PML IV but not markedly affected by PML-RARA (data not shown). These results indicate that PML-RARA specifically targets the interaction of PU.1 and PML IV.

We next considered the underlying mechanism for the differences in the effects of PML-RARA on PU.1 and AML1b. Immunoprecipitation experiments revealed that in PU.1 immunoprecipitates, the amount of p300 was remarkably reduced and that PML IV was lost when PML-RARA was coexpressed (Fig. 8D, top). In contrast, the coexpression of PML-RARA did not affect the amount of p300 coprecipitation but completely dissociated PU.1 from the PML IV immunoprecipitates (see Fig. S7C in the supplemental material). On the other hand, analysis of the AML1b complex revealed that the amount of p300 coprecipitation was not affected by the coexpression of PML I and/or PML-RARA (Fig. 8D, lower panels). The most striking difference was that PML-RARA coprecipitated with AML1b in the presence of PML I. Immunofluorescence analyses agreed with these results. PU.1 and AML1b were specifically colocalized in PML IV PODs and PML I PODs, respectively. When PML-RARA was coexpressed, the POD structures were disrupted, and PU.1 no longer colocalized with the PML IV microspeckles (Fig. 8E, top), whereas AML1b still colocalized with PML I microspeckles (Fig. 8E, bottom).

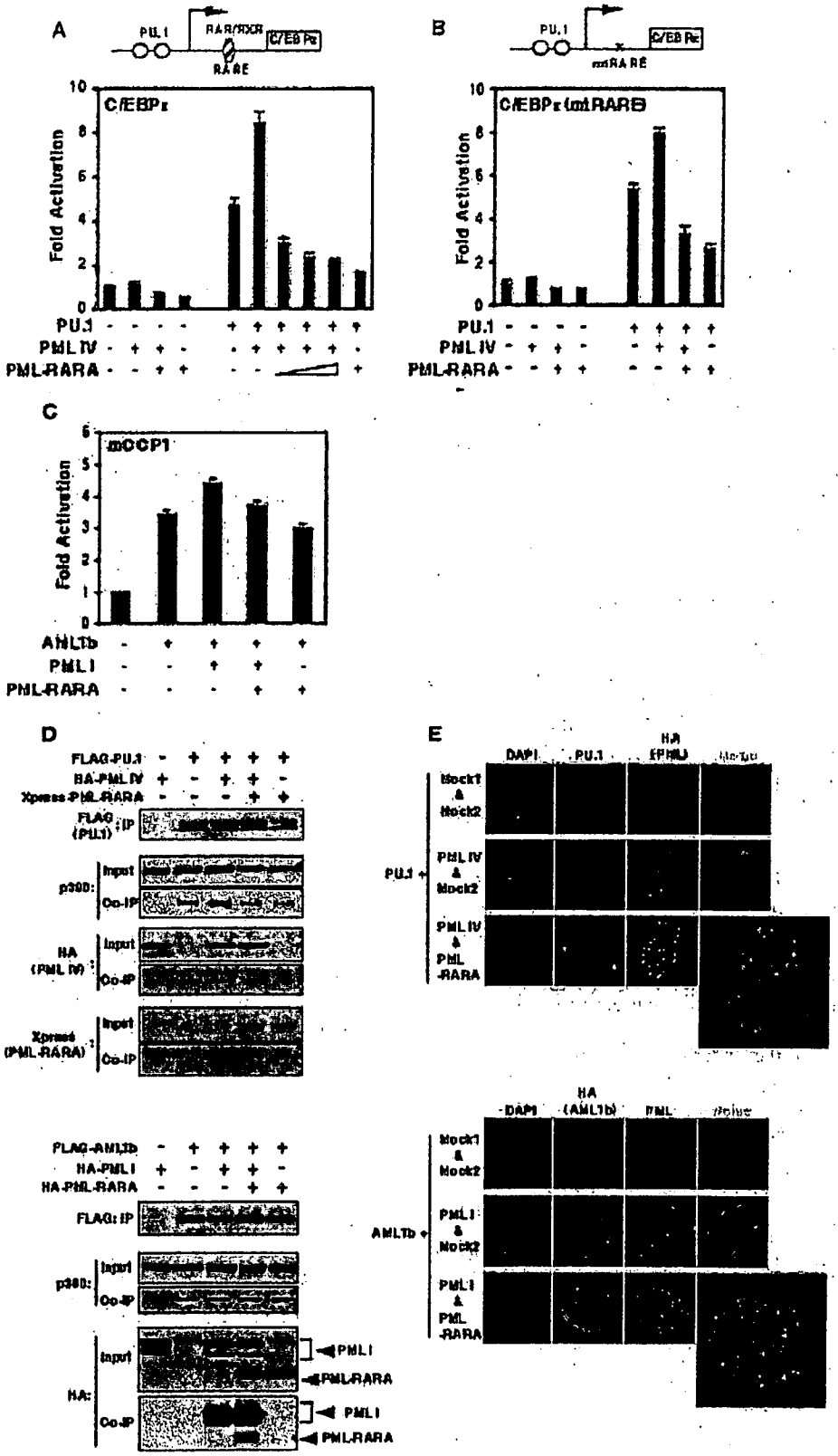
Finally, we examined the inhibition of PU.1-mediated granulocytic differentiation by PML-RARA in L-G/MT-PU.1 cells. The expression of PML-RARA in these cells (L-G/MT-PU.1/MT-PML-RARA) markedly suppressed PU.1-induced C/EBPε expression (Fig. 8F). The induction of PU.1 expression never reduced cell proliferation in those cells (data not shown). Morphological examination revealed that the expression of PML-RARA caused L-G cells to take on the appearance of APL cells and eliminated the ability of PU.1 to cause granulocytic differentiation, resulting in a premature arrest of differentiation (Fig. 8G).

DISCUSSION

In this study, we investigated the role of PML in myeloid differentiation and how the dominant-negative PML-RARA fusion affects the normal function of PML and gives rise to APL. Our results indicate the following: (i) PML cooperates with PU.1 to regulate C/EBPε expression during normal myeloid development, (ii) PML promotes the formation of an active transcription factor complex of PU.1 and p300 on the *C/EBPε* promoter during granulocytic differentiation, and (iii) PML-RARA has a dominant-negative effect not only on RA signaling but also on PML-induced transcription by disrupting the PU.1/PML/p300 ternary complex.

Role of PML in granuloid differentiation. PML is essential for RA action to induce terminal myeloid differentiation of precursor cells (32). On the other hand, the role of RA signaling during myeloid development is still controversial. Although C/EBPε is one of the most promising targets to help elucidate

FIG. 7. PML enhances the formation of the PU.1/p300 complex. (A) Expression of the PU.1/PML/p300 complex and selected C/EBP family members in HL-60 cells treated with RA for the indicated times. MW, molecular weight (in thousands). (B) PML and p300 are increasingly recruited onto the *C/EBPε* promoter in HL-60 cells treated with RA during the early stage of granulocytic differentiation. ChIP assays for region 2 were performed using antibodies as indicated. (C) The PU.1/p300 complex increases during RA-induced granulocytic differentiation. Lysates from HL-60 cells treated with RA for the indicated times were immunoprecipitated (IP) with an anti-PU.1 antibody (Ab), and coprecipitation of PML and p300 was analyzed by Western blotting. IgG, immunoglobulin G. (D) The PU.1/p300 complex increases along with PU.1 granulocytic differentiation. Lysates from L-G/MT-PU.1 cells treated with $ZnSO_4$ for the indicated time were immunoprecipitated with an anti-FLAG antibody (for PU.1 precipitation) and analyzed by Western blotting. The expression of C/EBPε and the PML protein is also shown. (E) Lysates from BOSC23 cells transfected with the indicated expression vectors were analyzed by immunoprecipitation with an anti-FLAG antibody and by Western blotting. (F) PML IV causes PU.1 and p300 to colocalize within PODs. Immunofluorescence was performed using anti-p300 and anti-PML (top) or an anti-PU.1 and anti-p300 (bottom) antibodies in NIH 3T3 cells transiently expressing PU.1 and PML IV. DAPI, 4′,6′-diamidino-2-phenylindole. (G) Functional relevance of PML IV effects on the PU.1/p300 complex. Luciferase assays using a reporter containing the *C/EBPε* promoter were performed using NIH 3T3 cells.



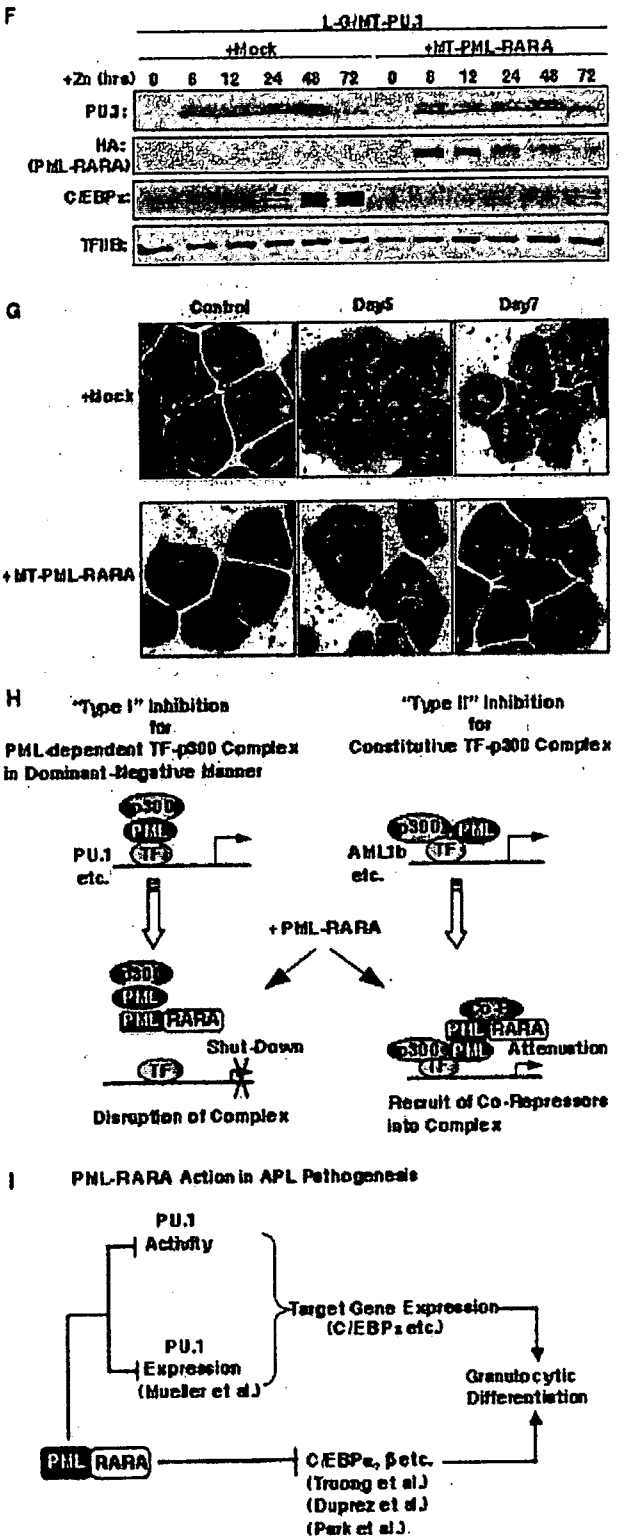


FIG. 8. PML-RARA disrupts PU.1/p300 complexes and prevents the enhancement of PU.1-induced C/EBPε expression by PML. (A) Luciferase assays in NIH 3T3 cells show that PML-RARA inhibits both the PU.1-mediated transactivation of *C/EBPε* and the enhancement of its transcription by PML IV in a dominant-negative fashion. (B) RARE is dispensable for PML-RARA-mediated inhibition of both PU.1- and PML IV-enhanced expression of *C/EBPε*. (C) PML-RARA has little effect on AML1b-mediated transcription. (D) PML-RARA disrupts the PML/PU.1/p300 complex but not the PML/AML1b/p300 complex. Lysates from BOSC23 cells transfected with the indicated expression vectors were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blotting. Note that a 20-fold-longer exposure was needed to detect the coprecipitation of p300 with PU.1 than was needed to detect the coprecipitation with AML1b. (E) Differential effects of PML-RARA on the POD colocalization of transcription factors. PML-RARA disrupts PODs, resulting in APL-associated microspeckle structures. PU.1 was lost from these structures, whereas AML1b remained. DAPI, 4′,6′-diamidino-2-phenylindole. (F) Western blots show that PML-RARA potently suppressed the ability of PU.1 to induce C/EBPε expression in L-G cells. (G) PML-RARA potently suppresses PU.1-induced granulocytic differentiation of L-G cells according to morphological criteria. (H) Model of the inhibitory mechanisms of PML-RARA towards different classes of transcription factor complexes. In type I inhibition (e.g., for the PU.1 complex), PML-RARA has a dominant-negative effect. In contrast, in type II inhibition (e.g., for the AML1b complex), PML-RARA only attenuates the activity. (I) Model of PML-RARA-mediated differentiation arrest.

the RA action in granulopoiesis (23), $RARA^{-/-}$ mice normally express C/EBPε and show normal granulopoiesis. Rather, neutrophil differentiation occurs faster for BM cells derived from $RARA^{-/-}$ mice than for those derived from wild-type mice (11). These observations suggest that RARA is dispensable for granulopoiesis, and so the role of PML is other than to modulate the RA signaling. Granulopoiesis seems to be controlled by two pathways, at least in vitro, finally accompanying the increased expression of C/EBP families (37). Insufficient granulopoiesis observed in $PML^{-/-}$ mice would be explained by the redundancy of the process itself or that of C/EBPβ and C/EBPε function in addition to the regulatory rather than the mandatory function of PML. Contrary to data from a previous study (32), we found that immature granulocytes increase in BM of $PML^{-/-}$ mice. This discrepancy may be due to a different set of antibodies used for flow cytometry, which successfully revealed this subtle difference, which was undetected by the differential counts on cytospin smears.

Since PU.1 expression can induce L-G cells to differentiate into mature granulocytes without any additional cytokines, it is likely that PU.1 activates an unrevealed transcription cascade(s) that directs terminal differentiation in a cell-autonomous manner. Although the Pβ promoter contains RARE, and *C/EBPε* is upregulated by a pharmacological dose of RA, at least in vitro (23), RA treatment does not affect the DNase I hypersensitivity of Pβ (14). Those findings imply that a *trans*-acting factor(s) other than the RAR would control the chromatin structure. Another interesting finding is that RA fails to induce the expression of C/EBPε in C/EBPα-deficient cells (37), suggesting that a C/EBPα-initiating transcription cascade is responsible for RA signaling. Since PU.1 is one of the target genes induced by C/EBPα (31), it would be reasonable to speculate on the possible involvement of PU.1 in the regulation of *C/EBPε*. Taking our results of *C/EBPε* Pβ promoter analysis together with in vivo observations of $RARA^{-/-}$ mice (11), we propose a model that RAR would be a negative regulator to allow transcription upon RA binding and that

PU.1 should instead be considered an authentic transactivator for *C/EBPε* transcription that mediates the instructive role of PU.1 in granulocytic differentiation.

Elucidation of PML and PU.1 interaction during granulocytic differentiation. Although each PML isoform resides within discrete subnuclear compartments, there have been few reports on their innate biological activities in myeloid development. In this study, overexpression experiments were employed to delineate the function of each PML isoform. Because their protein expression levels could not be equalized, it might be possible that PML II, III, and V isoforms could not represent significant synergistic action with PU.1 simply due to their insufficient availability. In addition, since the expression level of transduced PML IV seems to be high compared to that of the endogenous one, the effect of PML IV on PU.1 action should be carefully interpreted. On the other hand, we think that PML overexpression employed in this study may mimic, at least in part, the increase of PML isoforms during the early stage of terminal granulocytic differentiation. We observed that PU.1 and PML mutually regulate each other. Although the mechanism remains unclear, we speculate that the increase of PML expression during RA-induced granulocytic differentiation might be due, at least in part, to an increase of PU.1 expression. Although the issue of isoform change during differentiation seems to be very important, we think at present that during granulocytic differentiation, PML (and PML IV) is regulated mainly quantitatively. In turn, PML IV specifically associates with PU.1 in vivo and enhances its function. Thus, PU.1 autoregulates its own transcriptional capability. The isoform-specific interaction was closely linked to the functional cooperation of PML and PU.1. Note that the ternary complex formation of PU.1/PML/p300 on the *C/EBPε* promoter depends on PML recruitment and that it occurs rapidly after RA treatment, suggesting its role at the early stage of granulocytic terminal differentiation.

On the other hand, the relevance of the POD structure to transcriptional control remains elusive. Sumoylation of PML IV is a prerequisite for the normal architecture of PODs (39) and seems to be crucial for the transcriptional regulation of the PU.1/PML/p300 complex. Furthermore, the B boxes and coiled-coil domain of PML, essential for the formation of the normal POD structure, were required for the colocalization of PU.1 with PML IV. We also observed that PML VI cannot efficiently associate with PU.1 but does recruit it to PODs, and this activity is correlated with the cooperation of these proteins in the granulocytic differentiation of L-G cells, although PML VI does so less efficiently than PML IV. We speculate that PML VI indirectly regulates PU.1; e.g., PML VI may promote PML IV-mediated PU.1 targeting to PODs, although a complete understanding of the interaction between PML isoforms remains challenging. Another interesting finding is that PML IV augmented only the amount of C/EBPε expression but did not affect its time course profile in L-G cells expressing PU.1ΔPEST. Taken together with the finding that PU.1ΔPEST could not be efficiently recruited to PODs, we believe at present that PML IV-mediated ternary complex formation within the structurally integrated PODs would be required for the synergistic activation of transcription by the PU.1/PML/p300 ternary complex in vivo.

PU.1 and p300/CBP can directly interact, at least in vitro (33); however, we found that their association is rather weak compared with those of other transcription factors such as AML1b. We demonstrated that the PU.1/PML/p300 ternary complex is also formed on the target gene promoter and speculate that both PU.1 and p300 are efficiently assembled with the aid of PML IV, leading to the synergistic transcriptional activation of the target gene. As observed in LG/MT-PU.1 cells, efficient C/EBPε expression cannot be induced by PU.1 alone but requires the increase of PML expression and a possible reorganization of the PU.1 complex into an active form.

We also observed that PML IV increases PU.1 expression in both transient and stable coexpression systems throughout our experiments. Although its molecular mechanism remains to be elucidated, we speculate that the PML enhancement of PU.1 activity has an additional aspect of increased availability of PU.1 in addition to promoting the formation of the active transcription factor complex. Thus, PML IV seems to modulate PU.1 activity by both qualitative and quantitative mechanisms.

Reconsideration of the role of PML-RARA in APL: PML-RARA as a dominant-negative mutant causing dissociation of the PML-mediated transcription factor complex. Because RARA is a target for all APL-related chromosomal translocations, an alteration of its function must be required for promyelocytic transformation. PML-RARA has been thought to act as a dominant-negative inhibitor of the transactivator function of RARA. In addition, PML is a component of ligand-bound RARA complexes and regulates their activity (38). These observations have led to a proposed model in which PML-RARA acts as a dominant-negative inhibitor of the RA signaling pathway at multiple steps. On the other hand, since RARA has turned out to be dispensable for granulopoiesis, or rather acts as a transrepressor under physiological conditions in vivo (11), the enhanced repression of RA signals by PML-RARA would not likely be sufficient for fully elucidating the molecular mechanism of differentiation arrest in APL. It is not surprising, therefore, that the HDAC1-RARA fusion protein, a bona fide dominant-negative form of RARA, does not cause a block in myeloid differentiation in vivo and was not leukemogenic in those transgenic mice (18). Another study showed that homodimerizing artificial RAR fusions alone are poor initiators of leukemia, characterized by significant leukocytosis of mature neutrophils in vivo (28). Rather, the main role of the inhibitory effect of PML-RARA in RA signaling might only be priming for APL-like leukemia by the attenuation of spontaneous apoptosis (15). Therefore, the inhibitory roles of PML-RARA in normal PML function should be responsible for differentiation arrest.

We propose two different modes of action for PML-RARA inhibiting PML/transcription factor complexes (Fig. 8H). Because the association between PU.1 and p300 is weak, and largely depends on PML, PML-RARA heterodimerizes with PML and sequesters the PML/p300 complex from PU.1 (type I dominant-negative inhibition). On the other hand, AML1b still forms a stable complex with p300 regardless of the presence of PML I. In this case, PML-RARA gathers on the AML1b/p300 complex through heterodimerization with PML I and then attenuates the transcriptional activity, probably by recruiting corepressor complexes to overcome the histone acetyltransferase activity of p300/CBP (type II inhibition).

Thus, the inhibitory effects of PML-RARA would depend largely on the stability of a given transcription factor/p300 complex for PML.

Several lines of evidence suggest roles for C/EBPε in APL pathogenesis. Differentiation of BM cells from *C/EBPε*$^{-/-}$ mice is practically arrested at the promyelocyte stage, at least in vitro (34a). In addition, a previous excellent study shows that the overexpression of C/EBPε in APL rescues differentiation arrest in vitro as well as in vivo and prolongs the survival of mice transplanted with APL cells (29). On the other hand, repression of C/EBPε does not fully account for the pathophysiology of APL, because *C/EBPε*$^{-/-}$ mice do not capture the APL phenotype. Walter et al. previously reported that reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARA (30). Those authors also demonstrated that PML-RARA decreases the expression of PU.1 mRNA in PU.1-haploinsuficient mice by unknown mechanisms, causing the development of a hypomorphic PU.1 phenotype. Because PU.1 autoregulates its own expression (1), our results showing that PML-RARA inhibits the transcriptional capability of PU.1 agree with their findings and might partly explain the graded reduction of physiological PU.1 below a critical level, followed by the induction of myeloid leukemia (26). Thus, we suppose that the repression of PU.1 is one of the crucial mechanisms in PML-RARA leukemogenesis. Furthermore, PML-RARA is a multivalent suppressor for other C/EBP family members, including C/EBPα and C/EBPβ (4, 24, 29). We think that comprehensive inhibition of those transcription factors might be responsible for the full manifestation of APL.

There are four other types of APL-related chimeras that have been reported. Among them, NPM- or NuMA-RARA fusions do not affect the POD structure (8). In this respect, PML localization itself is not of primary importance for APL pathogenesis. On the other hand, disruption of the POD structure into microspeckles is invariably observed in t(15;17)-bearing APL, and the restoration of normal POD architecture is an early event in granulocytic differentiation following RA-induced degradation of PML-RARA (35). Therefore, POD structure-based PML function still seems to be a key target for the pathogenesis of PML-RARA-induced APL.

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ORIGINAL ARTICLE

Phosphorylation of PML is essential for activation of C/EBPε and PU.1 to accelerate granulocytic differentiation

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Promyelocytic leukemia (PML) is a nuclear protein that functions as a regulator of transcription, cell proliferation, apoptosis and myeloid cell differentiation. PML is subjected to post-translational modifications such as sumoylation and phosphorylation. However, the physiological significance of these modifications, especially for myeloid cell differentiation, remains unclear. In this report, we found that four serine residues in the PML C-terminal region are highly phosphorylated in a myeloid cell line. Wild-type PML accelerated G-CSF-induced granulocytic differentiation, but a phosphorylation-deficient PML mutant failed. PML interacted with C/EBPε, a transcription factor essential for granulopoiesis, activated C/EBPε-mediated transcription in concert with p300 and accelerated C/EBPε-induced granulocytic differentiation. Phosphorylation of PML was required for stimulating C/EBPε-dependent transcription and accelerating C/EBPε-induced granulocytic differentiation. We also found that PML phosphorylation was required for stimulation of PU.1-dependent transcription and acceleration of PU.1-induced granulocytic differentiation. These results suggest that phosphorylation plays essential roles in the regulation of PML to accelerate granulocytic differentiation through multiple pathways.

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Keywords: PML; phosphorylation; C/EBPε; PU.1; granulocytic differentiation

Introduction

Promyelocytic leukemia (PML) is a nuclear protein that plays a role in growth suppression, apoptosis, premature senescence and myeloid cell differentiation. PML concentrates in speckled subnuclear structures, termed PML nuclear bodies (NBs)/ND10/PODs, together with many other proteins, including Sp100, p53, pRb, Daxx and p300/CBP.[1] These facts suggest that PML plays a role in transcriptional regulation. The *PML* gene is involved in the chromosomal translocation t(15;17) and fuses to the retinoic acid receptor α (*RARα*) gene in the majority of cases of acute promyelocytic leukemia (APL), which is characterized by disruption of NBs into abnormal microspeckle structures.[2] In APL, the fusion gene product PML-RARα has been thought to block granulopoiesis by dominant-negative inhibition of both PML and RARα functions. PML is important for terminal differentiation of granulocytes, as shown by impaired granulopoiesis in PML-deficient mice.[3] Although PML plays a role in granulopoiesis, at least in part, by its modulation of the retinoic acid pathway,[3] it does not fully explain the role of PML in granulopoiesis, suggesting that other PML actions should be considered for myelopoiesis in the physiological condition.[4]

PML function is regulated by at least two distinct modifications, specifically, phosphorylation and sumoylation. Sumoylation is required for NB formation and enhancement of PML-dependent apoptosis.[5] Phosphorylation of PML is induced by ATR or Chk1/2 after DNA damage and it regulates p53-dependent and -independent apoptosis.[6,7] extracellular signal-regulated kinases (ERK)-mediated phosphorylation of PML increases sumoylation and enhances apoptosis in response to arsenic trioxde.[8] CK2-mediated phosphorylation leads to ubiquitin-dependent degradation of PML.[9] Thus, these two modifications are important for regulating PML-dependent apoptosis and PML stability. We previously reported that PML sumoylation might have an impact on granulocytic differentiation,[10] but the role of PML phosphorylation in regulating granulocytic differentiation has not yet been addressed.

Granulopoiesis is tightly controlled by lineage-specific transcription factors. CCAAT/enhancer-binding protein ε (C/EBPε) is expressed exclusively in granuloid cells and is essential for terminal differentiation of committed granulocyte progenitors.[10] Although C/EBPε can activate or repress target genes depending on its associated protein,[11] the essential partner in terminal granulocytic differentiation remains to be explored. PU.1 is also expressed exclusively in hematopoietic cells, and it is indispensable for the terminal differentiation of myeloid cells.[12] Recently, we reported that PML promotes the association of PU.1 with p300 to form the active transcriptional complex,[13] but the regulatory mechanism of their interaction remains to be elucidated.

L-G is an interleukin-3 (IL-3)-dependent myeloid cell line that can be differentiated into mature granulocytes in response to granulocyte-colony stimulating factor (G-CSF).[14] We found that PML is highly phosphorylated in L-G cells and the phosphorylation of PML is essential for accelerating G-CSF-induced granulocytic differentiation. We also found that PML associates with C/EBPε. PML activated C/EBPε-mediated transcription in cooperation with p300 and accelerated C/EBPε-induced granulocytic differentiation in a phosphorylation-dependent manner. These effects of phosphorylation on the PML-dependent regulation of granulopoiesis and transcription were also observed in the case of PU.1 regulation. Taken together, these findings suggest an essential role of PML phosphorylation in transcriptional regulation during the terminal differentiation of granulocytes.

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Materials and methods

Plasmids

The expression vectors for PML isoform IV, pLPCX-HA-PML and pLPCX-FLAG-PML, pMT-PU.1 and pLNCX–PU.1 were described previously.[10,13] C/EBPε cDNA encoding a 32-kDa protein was generated as described previously[15] and subcloned into pHM6, pLNCX and pMT vectors. Phosphorylation-deficit PML-4A or phosphorylation-mimic PML-4D mutants were generated by site-specific mutagenesis with overlapping extension PCR. Four serine residues at codons 505, 518, 527 and 530 were substituted to alanines or aspartic acids (TCC508SerGCC508Ala, -GAC505Asp; TCA518Ser-GCA518Ala, -GAC518Asp; AGC527Ser-GCC527Ala, -GAC527Asp; AGC530Ser-GCC503Ala, -GAC530Asp), respectively. The construction of sumoylation-deficient mutant PML-3R has been previously described.[10] A PML-dSP mutant lacking the serine- and proline-rich (SP) region (aa 502–554) was generated by appropriate restriction enzymes and PCR. All constructs were verified by DNA sequencing.

Construction of stable clones and retrovirus

First, 1×10^7 L-G cells were electroporated with pMT-C/EBPε or pMT–PU.1 plasmid, and stable clones were selected with 1 μg/ml of G418. Expression of C/EBPε or PU.1 was induced by adding 100 μM $ZnSO_4$ to the medium containing IL-3. Wild-type PML or its mutants were transduced by retrovirus infection as described previously,[10] and stable infectants were selected by 1 μg/ml of puromycin.

Identification of phosphorylation sites in the PML protein

FLAG–PML proteins purified from L-G cells were subjected to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis as previously described.[10] Phosphopeptides were identified using TurboSEQUEST software.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting analysis were performed as previously described.[10]

Antibodies

Primary antibodies used in this study were as follows: anti-FLAG (M2, Sigma, St Louis, MO, USA), anti-HA (3F10, Roche, Mannheim, Germany), anti-human C/EBPε (C-22, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human PML (1B9, MBL, Nagoya, Japan; H238, Santa Cruz Biotechnology), anti-human p300 (NM11, BD Bioscience, San Jose, CA, USA), anti-human PU.1 (T-21, Santa Cruz Biotechnology) and anti-mouse TFIIB (C-18, Santa Cruz Biotechnology).

Cells, in vitro *phosphatase treatment, immunofluorescence, luciferase reporter assay, quantitive reverse transcription PCR (qRT-PCR)*

Technical details are available in Supplementary Information.

Results

Identification of phosphorylation sites in PML protein

The primary structure of PML predicts putative phosphorylation sites within the N-terminal proline-rich (Pro) region and the C-terminal serine- and proline-rich (SP) region.[16] We first investigated the post-translational modification of PML stably expressed in L-G cells (Figure 1a). Western blot analysis showed that PML migrates with variable electrophoretic mobility. Four distinct bands were observed after the treatment of PML proteins with alkaline phosphatase (CIAP), indicating that PML is modified by phosphorylation as well as sumoylation in L-G cells.

To determine phosphorylation sites, exogenously expressed PML was purified from L-G cells and analyzed by LC/MS/MS. Four serines at codons 505, 518, 527 and 530 in the SP region of PML were identified as phosphorylation sites (Figure 1b). A mutant in which these serines were substituted to alanines (PML-4A) migrated to a similar position to that of phosphatase-treated wild-type PML, indicating that the four serine residues were mainly phosphorylated in L-G cells (compare Figures 1a and c).

Phosphorylation and sumoylation of PML are essential for acceleration of G-CSF-induced granulocytic differentiation

To elucidate the significance of PML phosphorylation and sumoylation in granulocytic differentiation, we also constructed phosphorylation-mimic PML-4D mutant with substitutions of serines 505, 518, 527 and 530 by aspartic acids, sumoylation-deficient PML-3R mutant with substitutions of lysines 65, 160 and 490 by arginines, or PML-dSP mutant with a deletion of the SP region containing the phosphorylation sites (Figure 1b). Then, we introduced these mutants as well as wild-type and PML-4A into L-G cells by retrovirus infection and tested their effects on the differentiation of L-G cells. Equivalent levels of wild-type and mutant PML proteins were expressed in L-G cells (Figure 1c). In the presence of IL-3, all of these infectants remained in immature myeloblasts (Figure 2a). After treatment with G-CSF for 5 days, an increased population of mature granulocytes was observed in PML-WT and PML-4D infectants when compared with vector-transduced cells (Figures 2a and b). However, the majority of PML-4A, -dSP and -3R infectants still remained at the myelocyte or metamyelocyte stage and only a small population of mature granulocytes was observed. To objectively evaluate the effects of PML mutants on cell differentiation, we used qRT-PCR to quantify the expression of neutrophil gelatinase (*NG*), a gene encoding a secondary granule protein which is upregulated in mature granulocytes (Figure 2c). Compared to vector-transduced cells, PML-WT and -4D, but not PML-4A, -dSP and -3R, enhanced the increase in expression of *NG* after treatment with G-CSF. These results indicate that, in addition to sumoylation, phosphorylation in the SP region is essential for PML to accelerate G-CSF-induced granulocytic differentiation.

PML associates with C/EBPε

Since PML is a transcriptional coregulator, the above results suggest that phosphorylation and sumoylation may be crucial for its regulatory action on some transcription factors involved in granulocytic differentiation. It has been demonstrated that C/EBPε functions during the G-CSF-induced granulocytic differentiation.[17] To examine the interaction between PML and C/EBPε, co-immunoprecipitation assays were performed. FLAG-PML and HA-C/EBPε were transiently coexpressed in Bosc23 cells, and immunoprecipitants with anti-FLAG antibody were analyzed by western blot with anti-HA antibody, showing co-precipitation of C/EBPε with PML (Figure 3a). Reciprocally, HA-PML was also co-precipitated with FLAG-C/EBPε. In HL60

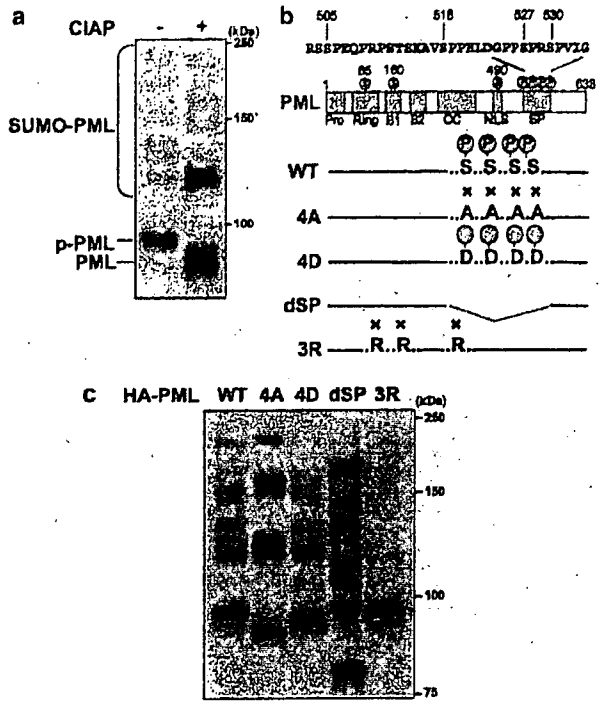

Figure 1 Covalent modifications of promyelocytic leukemia (PML) in granulocyte precursor cells. (**a**) Phosphorylation and sumoylation of PML in L-G cells. Stably expressed FLAG-PML was immunoprecipitated from the lysate of L-G cells, treated with (+) or without (−) CIAP and then analyzed by western blot with anti-FLAG antibody. Sumoylated, phosphorylated and unmodified PML are indicated. (**b**) Schematic diagrams of PML and PML mutants. Sites of phosphorylation and sumoylation are shown. Pro, proline-rich region; Ring, RING finger domain; B1 and B2, B boxes; CC, coiled-coil domain; NLS, nuclear localization signal; SP, serine- and proline-rich region. (**c**) Expression of each PML protein in stable L-G infectants. Total cell lysates from each PML infectant were analyzed by western blot with anti-HA antibody.

cells, endogenous PML and p300 were co-precipitated with C/EBPε whose expression was immediately increased after differentiation induced by all-*trans* retinoic acid (ATRA) treatment (Figure 3b). Notably, the amount of p300 that co-precipitated with C/EBPε was significantly increased within 2 days, demonstrating an accumulation of p300 in the C/EBPε/PML complex. To further confirm the association of C/EBPε and PML, HA–C/EBPε and PML were coexpressed in NIH3T3 cells, and double immunofluorescent staining was performed using anti-HA or anti-PML antibodies (Figure 3c). Without co-transfection of PML, C/EBPε dispersed throughout nuclei. When PML was coexpressed, C/EBPε accumulated in small dot-like structures, which coincided with NBs. Taken together, these results indicate that PML interacts with C/EBPε.

Essential role of PML phosphorylation for regulating C/EBPε activity

We generated an L-G/pMT-C/EBPε cell line, in which C/EBPε expression could be induced by exposure to $ZnSO_4$. The L-G/pMT-C/EBPε cells differentiated into mature granulocytes with segmented nuclei even in the presence of IL-3 within 6 days after exposure to $ZnSO_4$ (data not shown). To examine the effects of PML and its modifications on the C/EBPε-induced granulocytic differentiation, the cells were further infected with retroviruses encoding PML constructs or control vector, and then C/EBPε expression was induced (Figure 4a). The induced C/EBPε expression suppressed cell proliferation, which was enhanced by coexpression of PML-WT (Figure 4b). Compared to vector-transduced cells, an increased population of mature granulocytes was observed 4 days after PML-WT infectants were treated with $ZnSO_4$ (Figures 4c and d). Similarly, PML-4D inhibited cell proliferation and accelerated cell differentiation, but neither PML-4A nor -dSP did. Unexpectedly, PML-3R inhibited cell proliferation and accelerated cell differentiation as strongly as PML-WT. The increased expression of *NG* after $ZnSO_4$ treatment was enhanced by PML-WT, -4D and -3R, but not by PML-4A and -dSP (Figure 4e). A similar result was observed for the expression of lactoferrin (*LTF*), a gene that encodes a protein that is present in the secondary granules and is directly activated by C/EBPε.[18] These results indicate that PML accelerates C/EBPε-induced granulocytic differentiation and that phosphorylation, but not sumoylation, of PML is required for the effect.

We also examined whether the PML mutations affected the interaction and colocalization of PML with C/EBPε and p300 (supplementary figure). However, neither mutation affected these interactions and colocalizations. To test the effect of these modifications on C/EBPε-dependent transcription, we performed a luciferase reporter assay by co-transfecting plasmids for C/EBPε, p300 and wild-type or mutant PML together with a luciferase reporter containing the G-CSF receptor promoter (G-CSFR-luc), which contains a binding site for C/EBP family members (Figure 4f). While p300 alone modestly stimulated the transcriptional activity of C/EBPε, the coexpression of PML-WT further enhanced the C/EBPε-mediated transcription. PML-4D and -3R also stimulated transcription. However, PML-4A was

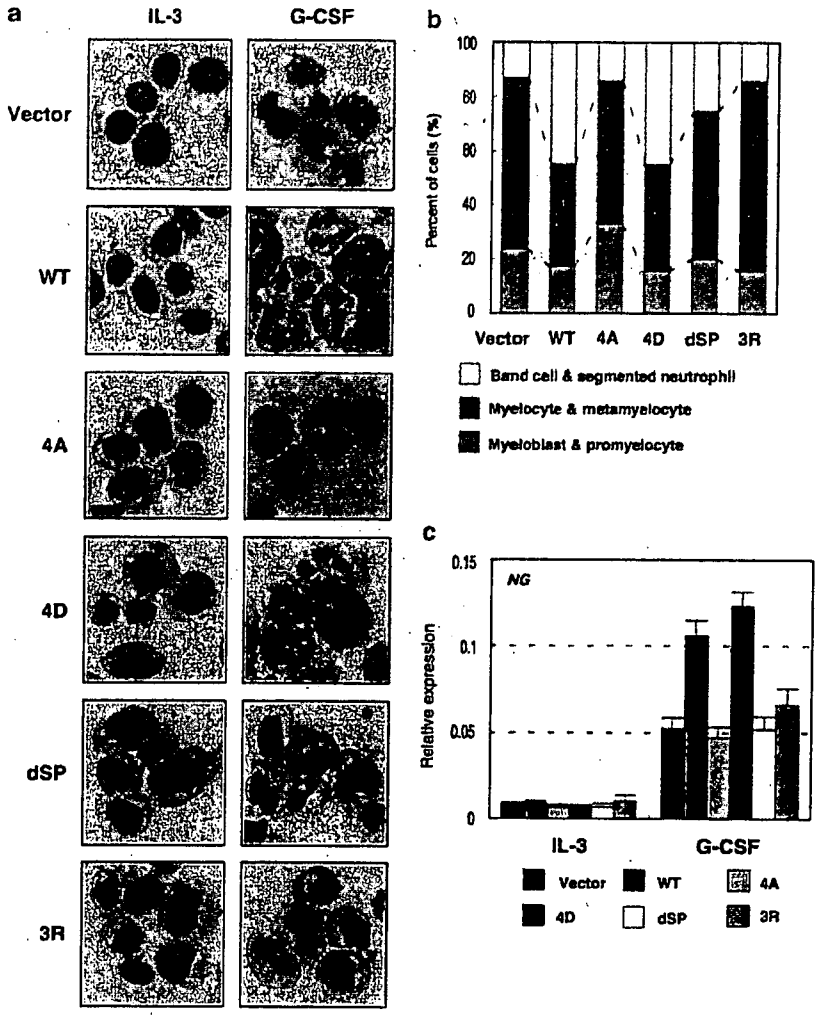

Figure 2 Phosphorylation and sumoylation of PML are essential for accelerating granulocyte-colony stimulating factor (G-CSF)-induced granulocytic differentiation. (a) Morphological evaluation of differentiation of L-G promyelocytic leukemia (PML) infectants treated with G-CSF for 5 days. (b) Differential count of L-G PML infectants after 5 days of treatment with G-CSF. (c) Comparison of secondary granule protein expression. Expression of neutrophil gelatinase (*NG*) in L-G PML infectants cultured in the presence of interleukin-3 (IL-3)- or G-CSF (for 3 days) was quantified by real time quantitative reverse transcription PCR (qRT-PCR). Data represent means ± s.d. of triplicate determinations of a representative experiment.

less potent, and PML-dSP was completely silent on the C/EBPε/p300-mediated transcription. It is particularly noteworthy that these effects of PML-WT and PML mutants on the C/EBPε-mediated transcription were correlated with their abilities to accelerate C/EBPε-induced granulocytic differentiation, suggesting that the activation of C/EBPε transcription by the phosphorylated, but not the sumoylated, form of PML plays an important role in granulopoiesis.

Requirement of phosphorylation for PML-dependent regulation of PU.1

Recently, we demonstrated that the transcriptional activity of PU.1 is also positively regulated by interaction with PML.[12] Therefore, we investigated the roles of PML modifications in PU.1-mediated transcription. A reporter assay showed that PML-WT, -4D and -3R activated PU.1-dependent transcription while PML-4A and -dSP did not (Figure 5a). To analyze the effects of PML modifications on PU.1-induced differentiation, we transduced PML constructs into L-G/pMT-PU.1 cells and then induced differentiation by $ZnSO_4$ treatment to express PU.1 (Figure 5b). PML-WT, -4D and -3R suppressed proliferation and accelerated granulocytic differentiation, whereas PML-4A did not (Figures 5c–e). The expression of *NG* was further increased in PML-WT, -4D and -3R infectants, but not PML-4A infectants, after treatment with $ZnSO_4$ (Figure 5f). These results indicate that PU.1-mediated transcription and granulocytic differentiation are also regulated by phosphorylated PML.

Discussion

PML accelerates granulocytic differentiation

One role of PML in terminal myeloid differentiation has been demonstrated in PML-deficient mice, which experience

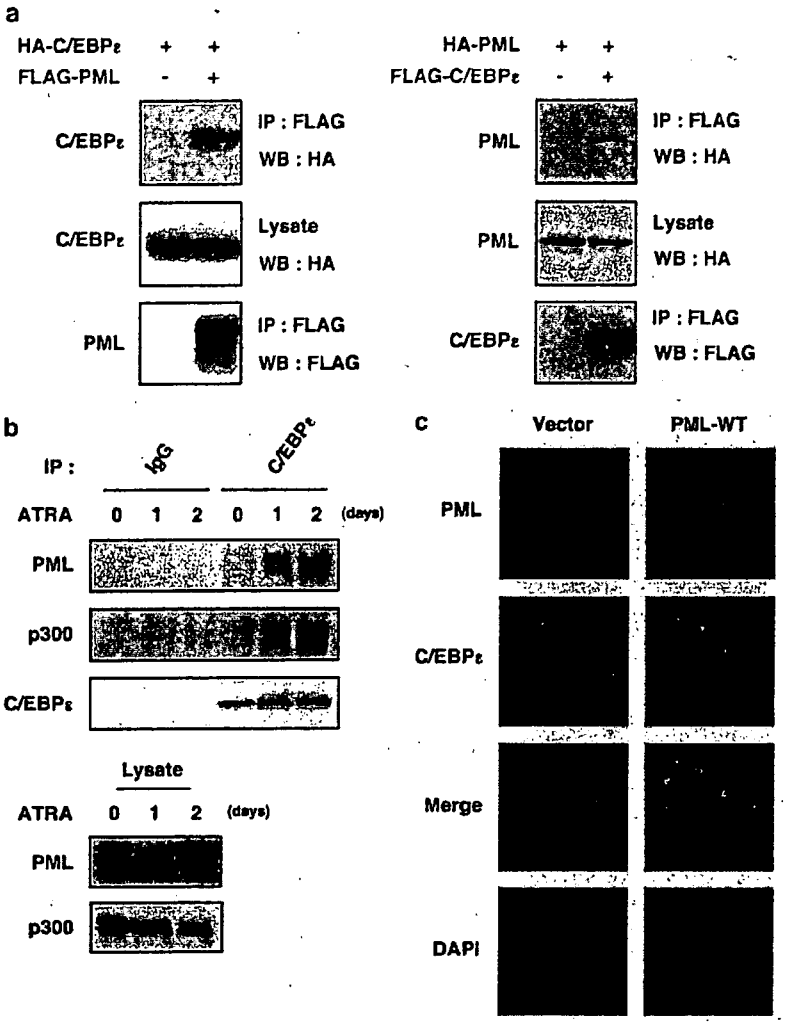

Figure 3 *In vivo* association of promyelocytic leukemia (PML) and C/EBPε. (a) Co-immunoprecipitation of PML and CCAAT/enhancer-binding protein e (C/EBPε). HA-C/EBPε and FLAG-PML were coexpressed in BOSC23 cells. Total expression (middle) or co-precipitated (top) C/EBPε was detected by western blot with anti-HA antibody. Immunoprecipitated PML was also analyzed with an anti-FLAG antibody (bottom) (left). A reciprocal experiment was also performed (right). (**b**) Association of endogenous PML and p300 to C/EBPε in HL60 cells. Cell lysates from HL60 cells treated with all-trans retinoic acid (ATRA) for the indicated days were immunoprecipitated with an anti-C/EBPε antibody and analyzed by western blot with anti-PML (top), anti-p300 (middle) and anti-C/EBPε antibodies (bottom),(upper panel). Levels of total PML and p300 in cell lysates were also analyzed (lower panel). (**c**) Colocalization of PML and C/EBPε within nuclear bodies (NBs). NIH3T3 cells were co-transfected with an expression vector for HA-C/EBPε together with either empty vector or FLAG–PML. C/EBPε was stained with anti-HA and FITC-labeled anti-rat antibodies. PML was stained with anti-PML and Texas red-labeled anti-rabbit antibodies. Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI).

impaired granulopoiesis.[3,13] In the present study, we found that PML accelerates G-CSF-induced granulocytic differentiation. A previous study[17] and our results (data not shown) demonstrate that G-CSF stimulation induces the expression of C/EBPε followed by granulocytic differentiation. These findings prompted us to determine whether PML regulates C/EBPε transcriptional activity to accelerate granulocytic differentiation. The current data illustrate that PML interacts with C/EBPε to activate its transcriptional activity and accelerates the granulocytic differentiation induced by overexpression of C/EBPε. Previously, we found that PML also accelerates PU.1-induced granulocytic differentiation.[13] Thus, PML appears to contribute to the regulation of granulopoiesis through interactions with C/EBPε and PU.1.

Phosphorylation of PML in myeloid cells

It has been suggested that the functions of PML are regulated at least in part by phosphorylation and sumoylation.[5–9] However, the role of PML phosphorylation in myeloid cell differentiation has not previously been addressed. In the present study, we found that four serine residues within the SP region of PML are highly phosphorylated in L-G cells. PML also contains several other serine residues in the N- and C-terminal regions that have been reported to be phosphorylated by ERK or CK2.[8,9] However, we did not detect these modifications by LC/MS/MS. Furthermore, alanine mutations of the phosphorylation sites did not affect the electrophoretic mobility of PML in L-G cells (data not shown). Thus, the SP region of PML is the main target of phosphorylation in L-G myeloid cells. While the upstream

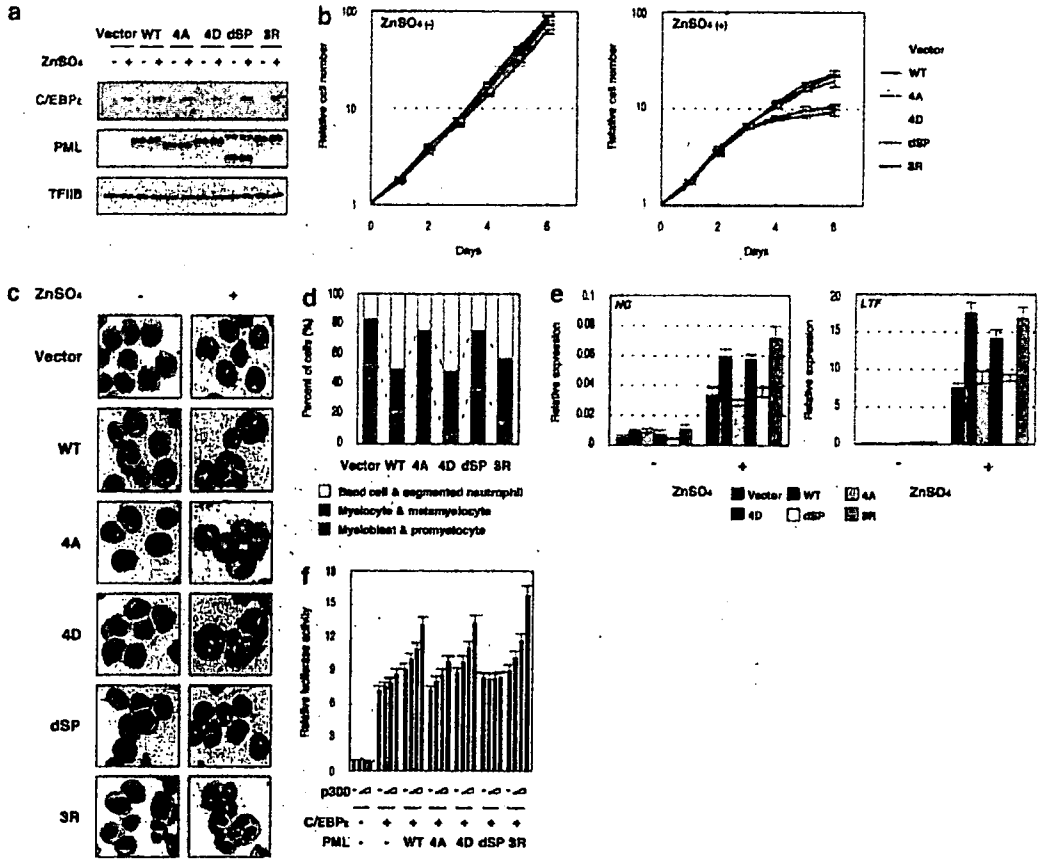

Figure 4 Effects of promyelocytic leukemia (PML) and PML phosphorylation on CCAAT/enhancer-binding protein e (C/EBPε)-induced granulocytic differentiation. (**a**) The expression of C/EBPε and PML in L-G/pMT-C/EBPε cells. Cells were cultured in the absence (−) or presence (+, for 24 h) of $ZnSO_4$. Total cell lysates were analyzed by western blot with anti-C/EBPε, -HA and -TFIIB antibodies. (**b**) Growth suppression of L-G/pMT-C/EBPε infectants by phosphorylated PML. Cells were cultured in the absence (left) or presence (right) of $ZnSO_4$. The relative number of viable cells is shown. The error bars represent the s.d. (**c**) Morphological evaluation of L-G/pMT-C/EBPε infectants cultured in the absence (−) or presence (+, for 4 days) of $ZnSO_4$. (**d**) Differential count of L-G/pMT-C/EBPε infectants. Cells were evaluated after 4 days of treatment with $ZnSO_4$. (**e**) Comparison of secondary granule protein expression. The expression of neutrophil gelatinase (*NG*) and lactoferrin (*LTF*) in L-G/pMT-C/EBPε infectants cultured in the absence (−) or presence (+, for 3 days) of $ZnSO_4$ was quantified by real time quantitative reverse transcription PCR (qRT-PCR). (**f**) Requirement of PML phosphorylation for cooperative activation of C/EBPε-mediated transcription with p300. NIH3T3 cells were transfected with the G-CSFR-luc reporter gene together with the indicated plasmids. The error bars represent the s.d.

kinase that phosphorylates PML during differentiation of L-G cells is unknown, kinases such as ERK and HIPK2, which phosphorylate serine residues within PxSP or SP sequences, interact with PML.[8,19,20] Since the overexpression of these kinases increases the phosphorylation of PML,[8,19] it is possible that they are involved in the phosphorylation of PML during the differentiation of L-G cells.

Role of PML modifications in granulocytic differentiation

In C/EBPε-induced granulocytic differentiation, we showed that the phosphorylation of PML is required for the acceleration of cell differentiation and the further increase in the expression of secondary granule protein gene including *LTF*, the product of a C/EBPε target gene. Although the mechanism by which PML regulates transcription is not sufficiently understood, it has been shown that PML promotes the interaction between transcription factors and coregulators such as p300.[10,13] In the present study, we found that p300 accumulates in the C/EBPε/PML complex during granulocytic differentiation. Despite the phosphorylation-independent association and colocalization of PML with C/EBPε and p300, the phosphorylation of PML is required for the synergistic effect of PML and p300 on the activation of C/EBPε-dependent transcription. Therefore, the phosphorylation of PML contributes to the acceleration of granulocytic differentiation, at least in part, by enhancing the effect of p300 on C/EBPε-dependent transcription.

The role of PML sumoylation in granulopoiesis remains unclear. In the present study, sumoylation of PML was not required for the acceleration of C/EBPε- and PU.1-induced granulocytic differentiation; however, sumoylation was required for induction by G-CSF, which suggests that the sumoylation of PML may contribute to the regulation of factors other than C/EBPε and PU.1 to accelerate G-CSF-induced granulocytic differentiation. These results suggest that G-CSF signaling induces cell differentiation through multiple PML-regulated pathways.

We conclude that both phosphorylation and sumoylation are essential for the ability of PML to accelerate granulocytic

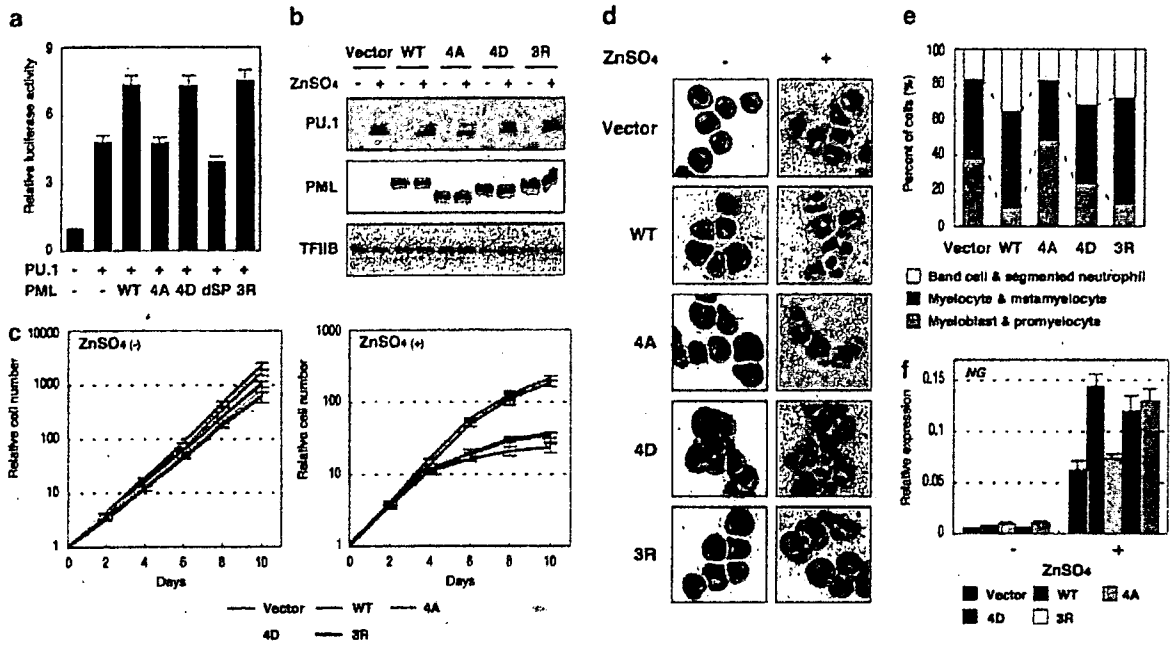

Figure 5 Effects of promyelocytic leukemia (PML) phosphorylation on PU.1-induced granulocytic differentiation. (**a**) Requirement of PML phosphorylation for activation of PU.1-mediated transcription. NIH3T3 cells were transfected with the C/EBPε-luc reporter gene together with indicated plasmids. (**b**) The expression of PU.1 and PML in L-G/pMT–PU.1 cells. Cells were cultured in the absence (−) or presence (+, for 24 h) of $ZnSO_4$. Total cell lysates were analyzed by western blot with anti-PU.1, -HA and -TFIIB antibodies. (**c**) Growth suppression of L-G/pMT–PU.1 infectants by phosphorylated PML. Cells were cultured in the absence (left) or presence (right) of $ZnSO_4$. The relative number of viable cells is shown. (**d**) Morphological evaluation of L-G/pMT–PU.1 infectants cultured in the absence (−) or presence (+, for 6 days) of $ZnSO_4$. (**e**) Differential count of L-G/pMT–PU.1 infectants. Cells were evaluated after 6 days of treatment with $ZnSO_4$. (**f**) Comparison of secondary granule protein expression. Expression of neutrophil gelatinase (*NG*) in L-G/pMT–PU.1 infectants cultured in the absence (−) or presence (+, for 3 days) of $ZnSO_4$ was quantified by real time quantitative reverse transcription PCR (qRT-PCR).

differentiation. Elucidating the regulatory mechanism of these modifications may help the development of therapeutic agents that induce differentiation of leukemia cells.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)