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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 (TCC508SerGC C508Ala, -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

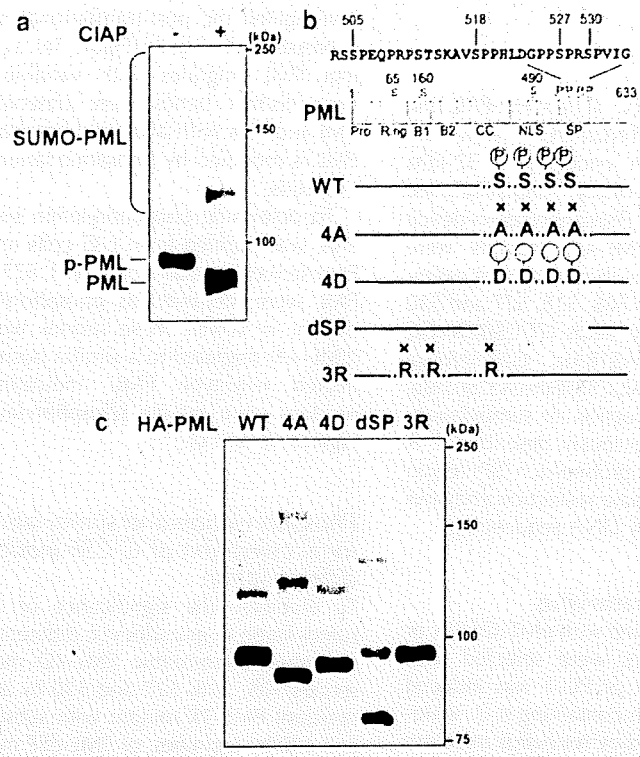

Figure 1 Covalent modifications of promyelocytic leukemia (PML) in granulocyte precursor cells. (**a**) Phosphorylation and sumoylation of PML in L-G cells. Stably expressed FLAG-PML was immunoprecipitated from the lysate of L-G cells, treated with (+) or without (−) CIAP and then analyzed by western 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

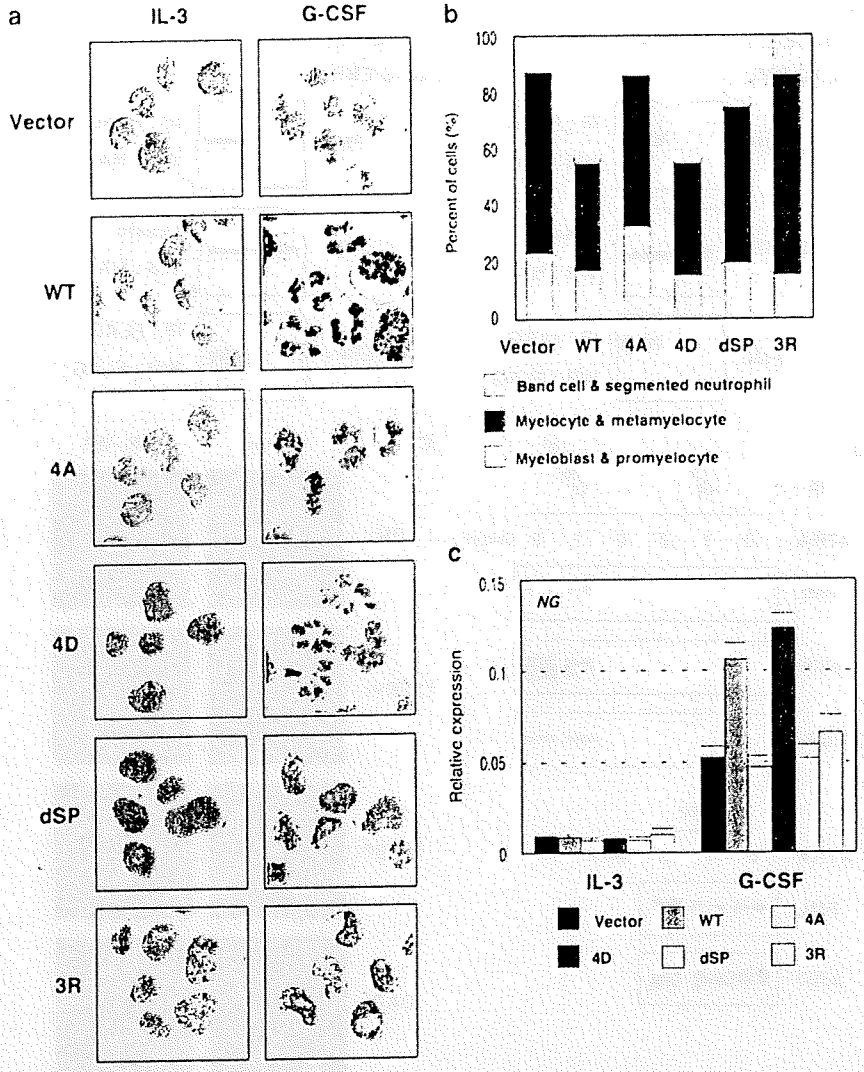

Figure 2 Phosphorylation and sumoylation of PML are essential for accelerating granulocyte-colony stimulating factor (G-CSF)-induced granulocytic differentiation. (**a**) Morphological evaluation of differentiation of L-G promyelocytic leukemia (PML) infectants treated with G-CSF for 5 days. (**b**) 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

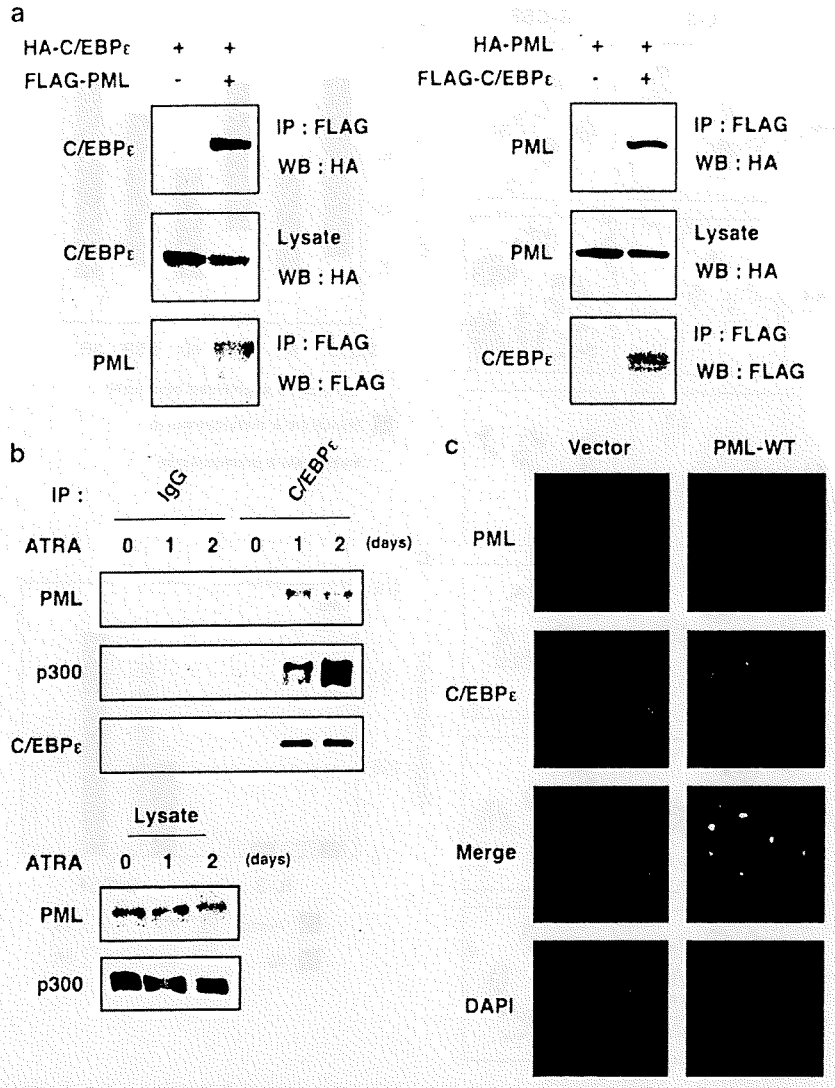

Figure 3 *In vivo* association of promyelocytic leukemia (PML) and C/EBPε. (**a**) Co-immunoprecipitation of PML and CCAAT/enhancer-binding protein e (C/EBPε). HA-C/EBPε and FLAG-PML were coexpressed in BOSC23 cells. Total expression (middle) or co-precipitated (top) C/EBPε was detected by western blot 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

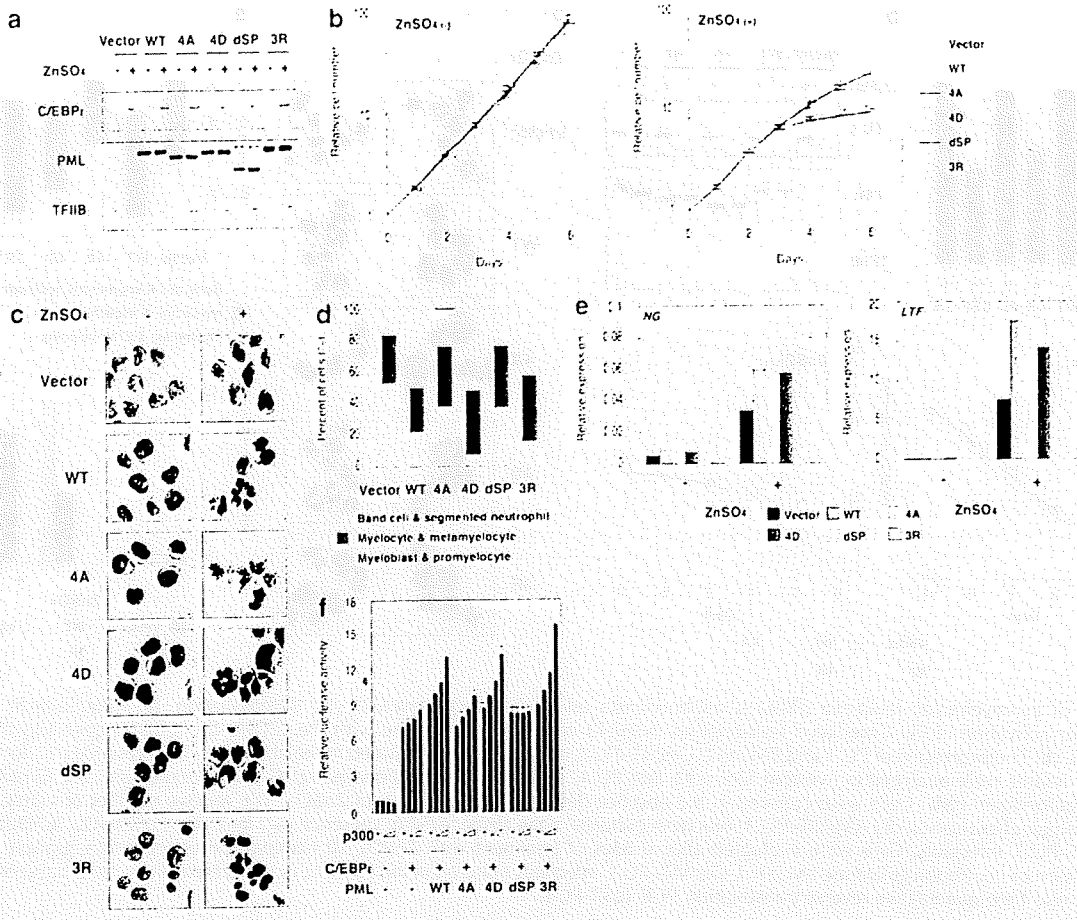

Figure 4 Effects of promyelocytic leukemia (PML) and PML phosphorylation on CCAAT/enhancer-binding protein e (C/EBPε)-induced granulocytic differentiation. (**a**) The expression of C/EBPε and PML in L-G/pMT-C/EBPε cells. Cells were cultured in the absence (−) or presence (+, for 24 h) of $ZnSO_4$. Total 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

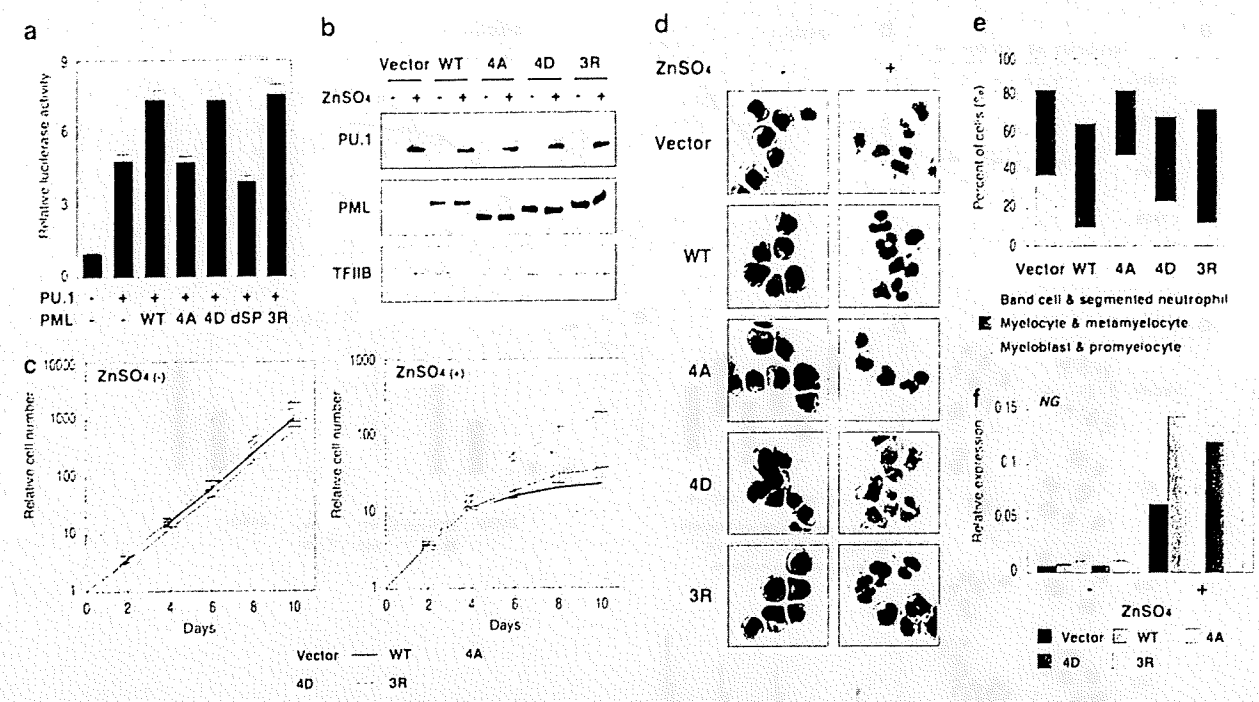

Figure 5 Effects of promyelocytic leukemia (PML) phosphorylation on PU.1-induced granulocytic differentiation. (**a**) Requirement of PML phosphorylation for activation of PU.1-mediated transcription. NIH3T3 cells were transfected with the C/EBPε-luc reporter gene together with indicated plasmids. (**b**) 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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ORIGINAL ARTICLE

Mutations of the *HIPK2* gene in acute myeloid leukemia and myelodysplastic syndrome impair AML1- and p53-mediated transcription

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The AML1 transcription factor complex is the most frequent target of leukemia-associated chromosomal translocations. Homeodomain-interacting protein kinase 2 (HIPK2) is a part of the AML1 complex and activates AML1-mediated transcription. However, chromosomal translocations and mutations of *HIPK2* have not been reported. In the current study, we screened mutations of the *HIPK2* gene in 50 cases of acute myeloid leukemia (AML) and in 80 cases of myelodysplastic syndrome (MDS). Results indicated there were two missense mutations (R868W and N958I) in the speckle-retention signal (SRS) domain of HIPK2. Subcellular localization analyses indicated that the two mutants were largely localized to nuclear regions with conical or ring shapes, and were somewhat diffused in the nucleus, in contrast to the wild type, which were mainly localized in nuclear speckles. The mutations impaired the overlapping localization of AML1 and HIPK2. The mutants showed decreased activities and a dominant-negative function over wild-type protein in AML1- and p53-dependent transcription. These findings suggest that dysfunction of HIPK2 may play a role in the pathogenesis of leukemia.
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Keywords: leukemia; HIPK2; mutation; AML1

Introduction

Leukemia is characterized by autonomous proliferation and impaired differentiation of hematopoietic precursor cells, and is considered to be the result of the accumulation of mutations. The mutations associated with the pathogenesis of leukemia include point mutations, gene rearrangements and chromosomal translocations. The *AML1* gene is the frequent target of leukemia-associated chromosome translocations, such as t(8;21)(q22;q22) (AML1-MTG8/ETO) (Miyoshi *et al.*, 1991), t(3;21)(q26;q22) (AML1-EV11/EAP/MDS1) (Nucifora *et al.*, 1993; Mitani *et al.*, 1994), t(16;21)(q24;q22) (AML1-MTG16) (Gamou *et al.*, 1998) and t(12;21)(p13;q22) (TEL-AML1) (Golub *et al.*, 1995; Romana *et al.*, 1995). AML1 protein forms a heterodimer with CBFβ and binds to the specific DNA sequence to regulate the expression of a number of hematopoietic genes (Meyers *et al.*, 1993; Ogawa *et al.*, 1993). Both AML1 and CBFβ are essential for the development of all definitive hematopoiesis lineages (Okuda *et al.*, 1996; Sasaki *et al.*, 1996; Wang *et al.*, 1996a, b; Okada *et al.*, 1998). AML1 forms complexes with PML and HATs, such as p300/CBP and MOZ, leading to the activation of transcription (Kitabayashi *et al.*, 1998, 2001a; Nguyen *et al.*, 2005). The genes encoding components of the AML1 complex are also the targets of leukemia-associated chromosomal translocations, including t(15;17) (q22;q11.2–q12) (PML-RAR α) (de The *et al.*, 1990, 1991; Kakizuka *et al.*, 1991; Kastner *et al.*, 1992), t(8;22)(p11;q13) (MOZ-p300) (Chaffanet *et al.*, 2000; Kitabayashi *et al.*, 2001b), t(11;22)(q23;q13) (E1A-p300) (Ida *et al.*, 1997), t(8;16)(p11;p13) (MOZ-CBP) (Borrow *et al.*, 1996), t(11;16)(q23;p13) (MLL-CBP) (Satake *et al.*, 1997; Sobulo *et al.*, 1997; Taki *et al.*, 1997) and inv(8)(p11q13)(MOZ-TIF2) (Carapeti *et al.*, 1998, 1999). AML1 mutations have been reported in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Osato *et al.*, 1999; Imai *et al.*, 2000; Preudhomme *et al.*, 2000). Recently, we found that homeodomain-interacting protein kinase-2 (HIPK2) was both involved in AML1 complex, and in the activation of the AML1 complex (Aikawa *et al.*, 2006). However, chromosomal translocations and mutations of *HIPK2* have not been reported previously.

HIPK2, as well as HIPK1 and HIPK3, is a member of a family of serine/threonine nuclear kinases and localizes to nuclear speckles (Kim *et al.*, 1998; Hofmann *et al.*, 2000; Wang *et al.*, 2001; Moller *et al.*, 2003a). Previous studies have shown there is binding of HIPK2 to various target proteins (Choi *et al.*, 1999; Hofmann *et al.*, 2003; Tomasini *et al.*, 2003; Zhang *et al.*, 2003, 2005; Moller *et al.*, 2003b; Rui *et al.*, 2004; Wiggins *et al.*, 2004), with subsequent modulation of the function of these proteins to corepressors or coactivators. HIPK2

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regulates gene transcription (Kim *et al*., 1998; Choi *et al*., 1999) inhibits cell growth (Pierantoni *et al*., 2001) and is involved in apoptosis (D'Orazi *et al*., 2002; Hofmann *et al*., 2002). HIPK2 activates p53 function, thereby promoting apoptosis following ultraviolet (UV) treatment (D'Orazi *et al*., 2002; Hofmann *et al*., 2002).

Human *HIPK2* has been mapped to chromosome 7q32–q34 (Hofmann *et al*., 2000; Wang *et al*., 2001), which is known to be frequently rearranged in AML, MDS, and in other human neoplasias (Mitelman *et al*., 1997). Moreover, HIPK2 expression is reduced in breast and thyroid carcinomas (Kim *et al*., 1999), thus suggesting HIPK2 as a candidate tumor suppressor gene (Kim *et al*., 1999). In the current study, we screened mutations of *HIPK2* in AML and MDS using denaturing high performance liquid chromatography (DHPLC). We found two missense mutations (R868W and N958I) within the speckle-retention signal (SRS) region of HIPK2 that were associated with the subcellular localization. These findings suggest HIPK2 may be involved in the pathogenesis of leukemia.

Results

Mutations of HIPK2 *gene*

In order to search for mutations in all coding exons of the *HIPK2* gene, we performed DHPLC analysis and direct sequence analysis. One missense mutation was detected in MDS, namely R868W in exon 12, and one missense mutation was detected in AML, namely N958I in exon 13 (Figure 1). The leukemic cells with these mutations showed normal karyotypes and no mutations in the *AML1* gene (data not shown). We also detected several single-nucleotide polymorphisms or nonsense mutations polymorphisms, none of which have been reported previously (Table 1).

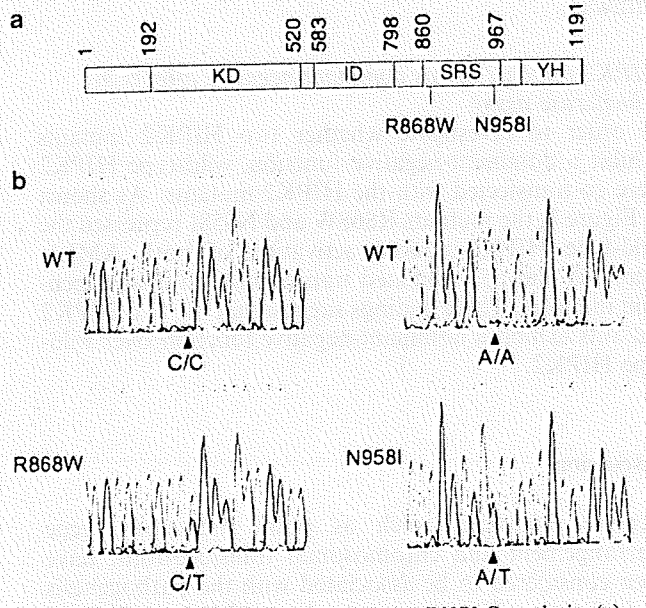

Figure 1 Screening HIPK2 mutations by DHPLC analysis. (a) Schematic representation of the domain structure of HIPK2 showing the location of the mutations identified in this study. KD, kinase domain; ID, interaction domain; SRS, speckle-retention signal; YH, tyrosine and histidine-rich domain. (b) DNA sequencing data of the two missense mutations. C→T was detected in MDS. A→T was detected in AML.

Subcellular localization of the HIPK2 mutants

The two mutations R868W and N958I were located within the SRS region, which is required for localization of HIPK2 in nuclear speckles (Kim *et al*., 1999, 2005; Engelhardt *et al*., 2003). This suggests that the mutations may affect the localization. To investigate subcellular localization, U2OS cells were transfected with wild type, R868W and N958I mutant of HIPK2. Subsequently, we performed immunofluorescence analysis (Figure 2). Wild-type HIPK2 was localized to the small nuclear speckles and these speckles were distributed within the nucleus. However, R868W and N958I mutants exhibited conical or ring shapes, and were diffused in the nucleus.

The HIPK2 mutants fail to colocalize with AML1b

In order to examine whether the HIPK2 mutants are colocalized with AML1b, U2OS cells were

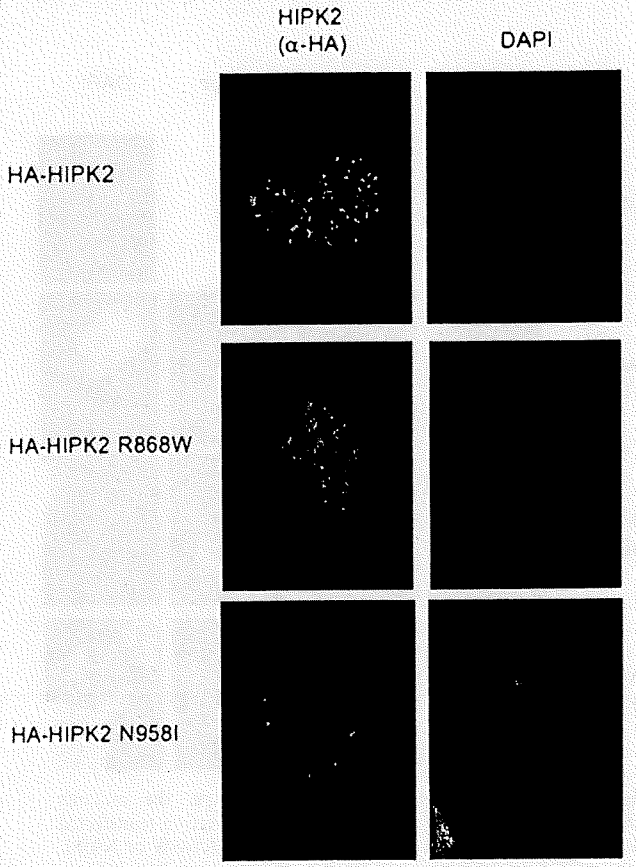

Figure 2 Subcellular localization of HIPK2 proteins (wild type, the mutants R868W and N958I). U2OS cells were transiently transfected with HA-tagged HIPK2 WT, R868W or N958I expression vectors alone. The expressed proteins were detected with anti-HA antibody. DNA was stained with DAPI.

co-transfected with FLAG-tagged AML1b and HA-tagged wild type or mutants of HIPK2 (Figure 3). Without cotransfection of HIPK2, AML1b was diffused in the nucleus. When coexpressed with HIPK2, AML1b was localized to the nuclear dot structures. Moreover, partial overlapping with wild-type HIPK2 was also observed. In contrast, the two mutants of the HIPK2 proteins, R868W and N958I, were not detected overlapping the localization of AML1b.

HIPK2 forms a ternary complex with AML1b and p300, and phosphorylates AML1b and p300 (Aikawa *et al.*, 2006). Therefore, we also investigated whether HIPK2 mutants can interact with and phosphorylate AML1b and p300 (Supplementary Figure 1). No significant differences in interaction or phosphorylation were observed between the wild type and the HIPK2 mutants.

Transcription activation abilities of the HIPK2 mutants

Our data suggest that HIPK2 plays an important role in AML1-mediated and p300-mediated transcription. (Aikawa *et al.*, 2006). To analyse effects of these mutants on transcription activation, reporter experiments were performed. As shown in Figure 4a and b, AML1-mediated and p300-mediated transcription was strongly activated by wild-type HIPK2, but not by the mutants R868W and N958I. These findings suggest that the two HIPK2 mutants lacked transactivation potential.

To determine if the SRS domain is required in order for HIPK2 to stimulate AML1-mediated transcription activation, a series of HIPK2 deletion mutants were constructed and tested for AML1-mediated transactivation. As shown in Figure 5, deletion of the C-terminal region to amino-acid position 1026 did not inhibit but rather stimulated the transactivation. However, further deletion to position 860 completely inhibited the transactivation. These results suggest that the SRS domain is required for HIPK2-mediated transactivation.

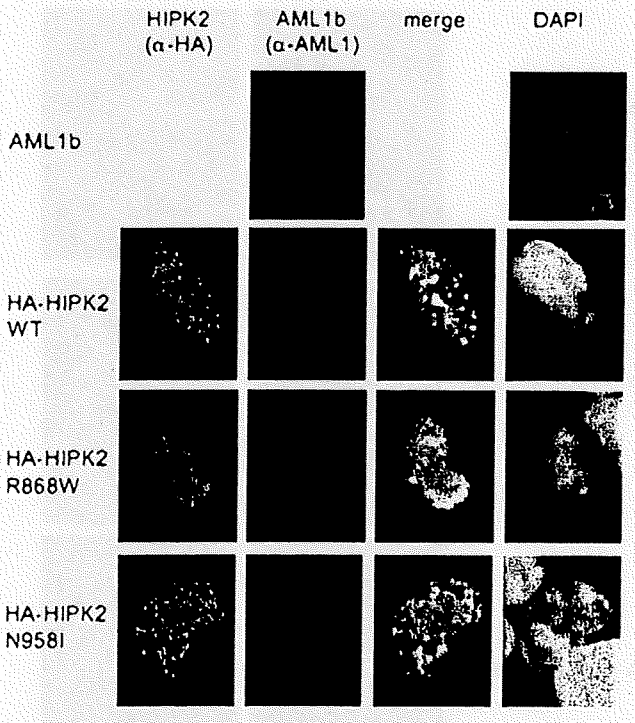

Figure 3 Colocalization of HIPK2 (wild type, the mutants R868W and N958I) with AML1b. U2OS cells were co-transfected with Flag-AML1b and HA-tagged HIPK2 WT, R868W or N958I. Cells were fixed 24 h later and proteins were detected by indirect immunofluorescence staining that used rabbit anti-AML1 and rat anti-HA as primary antibodies. Localization of AML1b is indicated by the red signal, and HIPK2 is indicated by the green signal. Overlapping localization is shown in yellow. DNA was stained with DAPI.

Effect of the HIPK2 mutants on p53 activity

HIPK2 phosphorylates p53 on Ser46, resulting in activation of p53-dependent transcription, cell growth regulation and apoptosis initiation (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002). To determine whether HIPK2 mutants modulate the transcriptional activity of p53, H1299 cells were co-transfected with the MDM2 promoter and expression vectors encoding p53 and HIPK2 (WT, KD, R868W, N958I). As shown in Figure 6a, p53-dependent transcription was strongly activated by wild-type HIPK2, but not by the mutants R868W and N958I.

To investigate whether HIPK2 affects DNA damage-induced expression of endogenous MDM2, MCF7 cells were transfected with HIPK2 (WT, KD, R868W, N958I) and were exposed to UV. As shown in Figure 6b, expression of MDM2 was increased by wild-type HIPK2, but not by the mutants R868W, N958I and KD. Taken together, these experiments suggest that two HIPK2 mutants affect the p53 pathway.

HIPK2 mutants act as dominant-negative effects in a luciferase assay

In order to investigate whether two HIPK2 mutants exhibit a dominant-negative function, wild-type HIPK2 were co-transfected with the HIPK2 mutants. As shown in Figure 7, the mutants R868W and N958I repressed the wild-type HIPK2-induced activation of both AML1-mediated and p53-mediated transcription in dose-dependent manners. These findings suggest that the two HIPK2 mutants exhibit a dominant-negative function over wild-type HIPK2.

Discussion

Genetic mutations, such as point mutations, gene rearrangement and chromosomal translocations, have been considered to be associated with the pathogenesis of leukemia. The genes encoding AML1 and/or its binding factors are frequent targets of the mutations or chromosome translocations associated with AML and MDS. We have found that HIPK2 is a part of the AML1 complex and is capable of stimulating AML1-mediated transcription activation (Aikawa *et al.*, 2006).

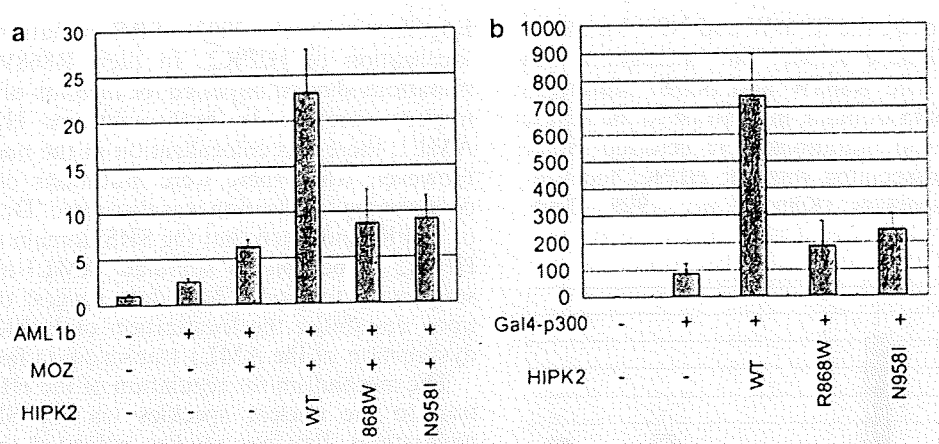

Figure 4 Effects of HIPK2 mutants on AML1-dependent transcription (**a**) and on transactivation by Gal4-p300 (**b**). (**a**) SaOS2 cells were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 250 ng of MOZ, 250 ng of wild type or the mutants R868W, N958I of HIPK2 and 2 ng of phRL-CMV. Cell lysates were prepared 24 h after transfection and were analysed for luciferase activity. (**b**) 293 T cells were transfected with 500 ng of pFR-luc (Gal4-luc), 100 ng of Gal4-p300, 400 ng of wild type or the mutants R868W, N958I of HIPK2 and 2 ng of phRL-CMV. Cell lysates were prepared 24 h after transfection and were analysed for luciferase activity.

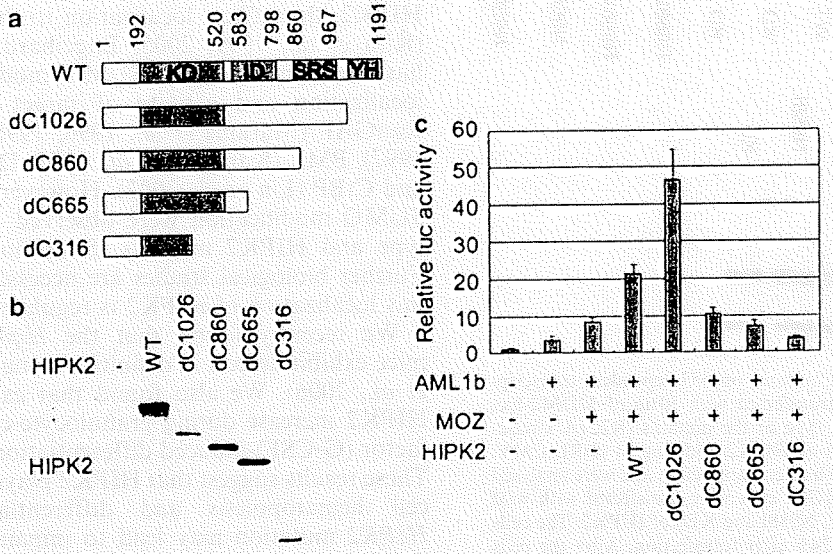

Figure 5 Effects of HIPK2 deletion mutants on AML1-dependent transcription. (**a**) Structure of HIPK2 deletion mutants. (**b**) Expression of HIPK2 mutants. 293T cells were transfected with HIPK2 mutants. Cell lysates were prepared 24 h after transfection and were analysed for their expression via immunoblot analysis. (**c**) Effects of HIPK2 mutants on AML1-mediated transcription activation. SaOS2 cells were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 250 ng of MOZ, 250 ng of wild type or deletion mutants of HIPK2 and 2 ng of phRL-CMV. Cell lysates were prepared 24 h after transfection and were analysed for luciferase activity.

These findings suggest that HIPK2 may be also the target of leukemia-associated mutations/chromosome aberrations. However, there have been no previous reports of mutation/chromosome abnormalities in the *HIPK2* gene. In the present study, we examined mutations of the *HIPK2* gene in 50 cases of AML and in 80 cases of MDS. Analyses indicated that there were two missense mutations (R868W and N958I) in the SRS domain of HIPK2. The *HIPK2* gene is mapped to the human chromosome 7q32–q34. Deletion of 7q is frequently found in AML and MDS. Initially, we expected mutation of *HIPK2* to occur in patients with the 7q deletion, thereby resulting in a homozygous loss of functional HIPK2. However, no mutations were found in these types of patients. As a matter of fact, patients with HIPK2 mutations showed normal karyotypes and did not have any other mutations in *AML1*. These results suggest that the mutation of HIPK2 plays a role similar to chromosome translocations and AML1 mutations in the pathogenesis of leukemia. However, further analysis is required to confirm this hypothesis.

Recently, we found that HIPK2 interactions result in the phosphorylation of AML1 and p300 leading to stimulation of AML1-mediated transcription (Aikawa *et al.*, 2006). The two missense mutants that we identified here (R868W and N958I) showed decreased activities and a dominant-negative function over wild-type protein in AML1- and p53-dependent

transcription. However, the R868W and N958I mutants affect neither the kinase activity nor interaction with AML1 and p300. The R868W and N958I mutations were found in the SRS domain, in contrast to the kinase domain, for which no mutations were detected. Since SRS is reportedly associated with the HIPK2 localization to nuclear speckles (Kim *et al.*, 1999, 2005; Engelhardt *et al.*, 2003). SRS mutations may affect localization of HIPK2. In fact, R868W and N958I mutations showed impaired localization of HIPK2 in the nuclear speckles. In both wild-type HIPK2 and in AML1, there was colocalization in the nuclear speckles. However, when there were mutations of HIPK2, the overlapping localization was disrupted. Deletion analysis of HIPK2 indicated that the SRS domain is required for HIPK2 to be able to stimulate AML1-mediated transcription activation. These data suggest that the mutations destabilize the localization of HIPK2, leading to dysfunction of the AML1 transcription factor complex.

The mechanism for regulation of the HIPK2 localization to the nuclear speckles via the SRS is poorly understood. Previous studies indicated that HIPK2 is modified by a small ubiquitin-like modifier 1 (SUMO-1) (Kim *et al.*, 1999), and that SUMO modification of HIPK2 occurs at lysine 25 (Hofmann *et al.*, 2005; Sung *et al.*, 2005). The SRS contains a domain that interacts with a SUMO-conjugating (E2) enzyme, mUBC9 (Kim *et al.*, 1999). When there is SUMO modification of HIPK2, this affects localization to the nuclear speckles (Kim *et al.*, 1999, 2005; Engelhardt *et al.*, 2003). It has been reported that SUMO modification regulates localization and activity of target proteins such as RanGAP (Matunis *et al.*, 1996, 1998; Mahajan *et al.*, 1997), PML (Kamitani *et al.*, 1998; Duprez *et al.*, 1999) and CtBP (Lin *et al.*, 2003). However, no differences in SUMO modification were observed between the wild-type and HIPK2 mutants (Supplementary Figure 2). Further biological studies are necessary to clarify how this localization of HIPK2 is regulated by SRS.

We recently found that the *Hipk1/Hipk2*-deficient mice exhibit defects in definitive hematopoiesis (Aikawa *et al.*, 2006). We also found that expression levels of HIPK2 increase during granulocyte-colony stimulating factor (G-CSF)-induced differentiation of myeloid cells. These results suggest that HIPK2 plays a role in myeloid cell hematopoiesis and differentiation. Therefore, *HIPK2* mutation may lead to impairment of hematopoiesis and/or differentiation of myeloid cells, resulting in the pathogenesis of leukemia.

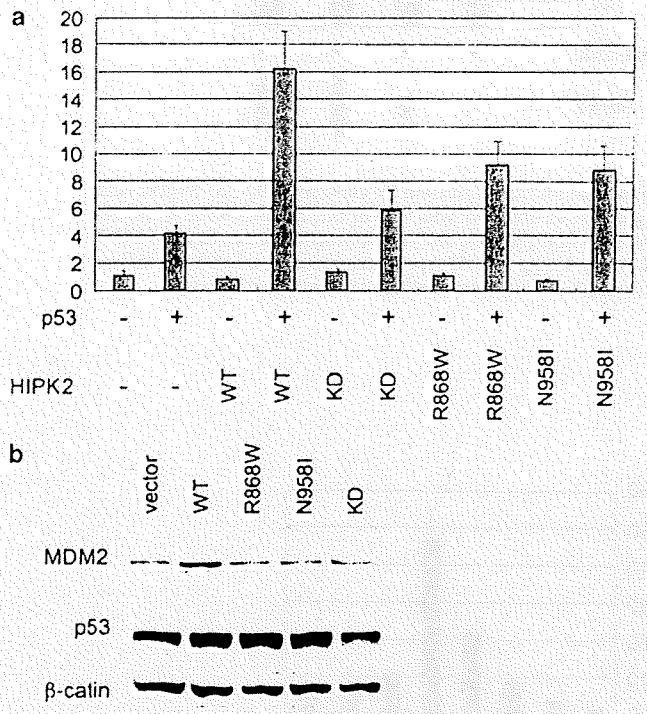

Figure 6 Effects of HIPK2 mutants on p53-dependent transcription. (**a**) H1299 cells were transfected with 50 ng of MDM2-luc, 2.0 ng of p53, 70 ng of wild type or the mutants (K221A kinase-dead (KD), R868W, N958I) of HIPK2 and 2 ng of phRL-CMV. Cell lysates were prepared 24 h after transfection and were analysed for luciferase activity. (**b**) MCF7 cells were transfected with wild type or the mutants (R868W, N958I and KD) of HIPK2. The cells were exposed to 30 J/m^2 at 24 h after transfection. After the cells were cultured for 8 h, cell lysates were prepared and analysed by immunoblotting.

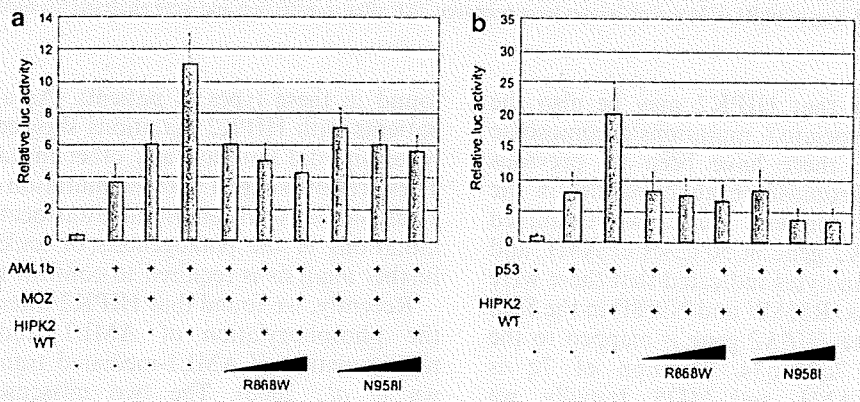

Figure 7 Dominant-negative effects of HIPK2 mutants on wild-type HIPK2 activity. (**a**) SaOS2 cells were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 250 ng of MOZ, 250 ng of wild type, and mutants R868W, N958I of HIPK2. (**b**) H1299 cells were transfected with 50 ng of MDM2-luc, 2.0 ng of p53, 70 ng of wild type and the mutants R868W, N958I of HIPK2. Cell lysates were prepared 24 h after transfection and were analysed for luciferase activity.

Inactivation of the p53 pathway is associated with cancer development. Although p53 mutations are observed in more than 50% in solid tumors, p53 mutations are found in only 17 and 10% of AML and MDS cases, respectively (Krug *et al*., 2002), suggesting that leukemia can develop even in the presence of normal p53. In fact, no mutations in p53 were detected in two patients, with *HIPK2* mutations. It has been reported that HIPK2 is involved in induction of p53-dependent transcription, cell cycle and apoptosis (D'Orazi *et al*., 2002; Hofmann *et al*., 2002). In this study, we found that HIPK2 mutants exhibited impaired activation of p53-mediated transcription as well as AML1-mediated transcription. Thus, we believe that HIPK2 mutations affect both AML1-mediated cell differentiation and p53-mediated apoptosis to induce leukemia.

In summary, within the SRS, we found two mutations of the *HIPK2* gene, R868W and N958I, which are associated with the subcellular localization of HIPK2. These mutations impaired overlapping localization of AML1 and HIPK2, as well as the activation of AML1-mediated transcription by HIPK2. Furthermore, the two mutants decreased p53-mediated transcriptional activity. Therefore, our results provide new evidence on a possible mechanism for the pathogenesis of leukemia.

Materials and methods

Patient samples

We examined 80 cases of MDS (31 refractory anemia with excess blasts (RAEB), 19 RAEB in transformation (RAEBt), 30 AML following MDS) and 50 cases of AML without antecedent MDS (4 M0, 13 M1, 9 M2, 9 M4, 12 M5, 2 M6, 1 M7) for screening mutations of *HIPK2*. All of these patients were diagnosed by morphology, immunophenotype, karyotype and AML1 mutation analysis as described previously (Harada *et al*., 2004). Patients with t(15;17), t(8;21), inv(16) or AML1 point mutation were excluded from this analysis. Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University.

Screening for HIPK2 *mutations*

Genomic DNA was isolated, and genomic fragments were amplified by PCR using 18 sets of primers (Table 2). The PCR reaction was performed for 35 cycles in a 10 μl mixture containing genomic DNA, 1 μl of 10 × PCR buffer, 0.25 μl of 10 mM dNTP, 2 pmol of each primer and 0.07 U *Taq* polymerase. Each PCR cycle consisted of 94°C for 20 s, 56–60°C for 40 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gels. Mutation screening for *HIPK2* was carried out by prescreening with DHPLC analysis (WAVE DNA Fragment Analysis System, Transgenomic, Omaha, NE, USA). If an abnormal peak sample was detected, it was directly sequenced using a Big Dye Terminator Sequencing kit and ABI Prism 3100 Genetic Analyzer.

Cell culture and transfection

SaOS2, 293T, U2OS, MCF7 and H1299 cells were cultured in Dulbecco's modified Eagle's medium or RPMI supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. U2OS and MCF7 cells were transfected by Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the reporter assay, 293T, SaOS2 and H1299 cells were transfected by the calcium phosphate precipitation method.

Plasmids

The human AML1b expression vector pLUCX-FLAG-AML1b, human p300 expression vector pLUCX-FLAG-p300, human MOZ expression pLUCX-FLAG-MOZ, human HIPK2 expression vector pLUCX-HA-HIPK2, pLUCX-HA-HIPK2 KD, human p53 expression vector pLUCX-FLAG-p53, MDM2-luc and MPO-luc have been described previously (Kitabayashi *et al*., 2001a; Aikawa *et al*., 2006). The expression vectors of the HIPK2 mutants, LUCX-HA-HIPK2 R868W, LUCX-HA-HIPK2 N958I, were generated by site-specific mutagenesis using overlapping extension PCR. The sequences of these constructs were checked by DNA sequencing.

Table 1 Mutations and SNPs of *the HIPK2* gene in AML and MDS

	Nucleotides		*Amino acids*		*Populations*	
	Positions	*Changes*	*Positions*	*Changes*	*AML*	*MDS*
Missense mutations						
Exon12	2602	C to T	868	R to W		1/80 (1.25%)
Exon13	2873	A to T	958	N to I	1/50 (2%)	
SNPs						
Exon2	891	C to T			8/50 (16%)	19/80 (23.75%)
Exon2	1059	C to G			15/50 (30%)	30/80 (37.5%)
Exon6	1557	C to T			1/50 (2%)	2/80 (2.5%)
Exon10	2145	G to A				1/80 (1.25%)
Exon12	2682	G to T			1/50 (2%)	
Intron3	−56 from exon 4	A to G				2/80 (2.5%)
Intron4	−4 from exon 5	G to A			1/50 (2%)	
Intron10	−9 from exon 11	G to C				1/80 (1.25%)
Intron13	−4 from exon 14	G to A				1/80 (1.25%)

Abbreviations: SNPs, single-nucleotide polymorphisms; *HIPK2*, homeodomain-interacting protein kinase-2; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.

Table 2 The primer sets for DHPLC for the *HIPK2* gene

Exons	*Forward primers*	*Reverse primers*
Exon2a	GTACCAAGGTGACTCCGATACTT	CAGTGCTTCGACGCATTAGGTTG
Exon2b	CAGCCACCACAACCGTCAG	CGTTTCCAGCACTTGACCACT
Exon2c	CTGTCTCCTTGTGGACGGTCT	ATGTTGGAGCAGAACCTCTATGACT
Exon2d	GTTTGAGGTCAGCGTGGATAA	GCCACTGCCACCACGTCTAC
Exon3	GCTTCCTGCATTTCCAACTTGCT	CAATGAAATGCTAATCCAGGCTA
Exon4	CTCAAAGCCAGCAGAGCCATTAG	TCCCTAAGCGCTGGGCCACT
Exon5	TGGGAAAGAGACCCTCTGTGAGA	CAGAAGTCTTGCTGCCCTTGGTT
Exon6	TCAGGAGGAAGTTACAGGGCAAA	CTTTAAAGTTTGCCGATCCCTGG
Exon7	CATCCCACGTTACCCTCTTCCC	AGTCCTGAGGCTCACACTG
Exon8	CCAACATGCCACCTCCCTCATTT	CAGAGGCCGTTCTGTAAGAAGCA
Exon9	AGCTGTTCACAGCGTCTCAGCTA	TAGGGAGAGAGGGAGTGGAGATA
Exon10	ATCAATTATGTGATTAGCAAACATGGTC	ACGTGCCTCCCATCCCAGAC
Exon11	GGAAGCTTGATCTTATAGAGGAG	CATTTCTTTAGCACTCACATCCC
Exon12	GGGCCTGACCTTCCTCTGTG	CCTCCCGGCTCCTTCCTG
Exon13	CTCACGGCCTTCTCCCACCT	TACAGCAACATTTCTAGCAGCAG
Exon14	GCTGGGACCCTGCCACTGAT	CTCTGAAGCGGAAGGATGAGGG
Exon15a	CTCCTCCCATTCCTTCCTCCC	GTGGGTATCCAGTGTAGACGGTG
Exon15b	CCACCATCCACCCGAGTCAG	GAATGGGTTCTTGAGCTGGGTTT

Abbreviations: DHPLC, denaturing high performance liquid chromatography; *HIPK2*, homeodomain-interacting protein kinase 2.

Immunofluorescence analysis

U2OS cells were grown in four-well chamber slides and co-transfected with various expression vectors. Twenty-four hours after being co-transfected, cells were fixed for 10 min with 4% paraformaldehyde at room temperature, then permeabilized with 0.1% Triton-X 100 in phosphate-buffered saline (PBS) for 10 min. Next, cells were washed three times with PBS and blocked with 0.2% FCS at 4°C for overnight. Cells were incubated with primary antibodies in PBS containing 0.2% FCS for 1 h at room temperature. Cellular nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). Images under a fluorescence microscope (Olympus, Melville, NY, USA) were captured with a CoolSNAP-HQ CCD camera (Roper Scientific, Ottobrunn, Germany) at a magnification of ×60.

Luciferase assay

SaOS2, 293T or H1299 cells were co-transfected with various expression vectors in 24-well plates, and luciferase activity was assayed after 24 h using a luminometer Lumat LB9507 (Berthold, Bad Wildbad, Germany) according to the manufacturer's protocol (Promega, Madison, WI, USA). Results of reporter assays represent the average values for relative luciferase activity generated from three independent experiments that were normalized using the activity of the enzyme from phRL-CMV as an internal control.

Immunoprecipitation, immunoblotting and antibodies

For immunoprecipitation experiments, cell were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate pH 7.0, 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride and protease inhibitor. Cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads (Sigma, St Louis, MO, USA) and slightly rotated at 4°C overnight. The absorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by FLAG peptide and dissolved with the same volume of 2× sodium dodecyl sulfate (SDS) sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence using ECL plus Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). The primary antibodies used in this study were anti-FLAG (M2) (Sigma), anti-HA (3F10) (Roche, Basel, Switzerland), anti-human AML rabbit polyclonal, and anti-p300 rabbit polyclonal (N15) antibodies.

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PML-Retinoic Acid Receptor α Inhibits PML IV Enhancement of PU.1-Induced C/EBPε Expression in Myeloid Differentiation▽†

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PML and PU.1 play important roles in myeloid differentiation. PML-deficient mice have an impaired capacity for terminal maturation of their myeloid precursor cells. This finding has been explained, at least in part, by the lack of PML action to modulate retinoic acid-differentiating activities. In this study, we found that C/EBPε expression is reduced in PML-deficient mice. We showed that PU.1 directly activates the transcription of the *C/EBPε* gene that is essential for granulocytic differentiation. The type IV isoform of PML interacted with PU.1, promoted its association with p300, and then enhanced PU.1-induced transcription and granulocytic differentiation. In contrast to PML IV, the leukemia-associated PML-retinoic acid receptor α fusion protein dissociated the PU.1/PML IV/p300 complex and inhibited PU.1-induced transcription. These results suggest a novel pathogenic mechanism of the PML-retinoic acid receptor α fusion protein in acute promyelocytic leukemia.

Acute promyelocytic leukemia (APL) has been characterized as a differentiation arrest at the promyelocyte stage due to t(15;17) reciprocal chromosomal translocation that generates a PML-retinoic acid (RA) receptor α (RARA) fusion protein (3, 10). All-*trans* RA (ATRA) induces the differentiation and elimination of APL clones (35). Biochemical evidence that RARA and PML-RARA are bidirectional transactivators (12) and the participation of the *RARA* gene in all APL syndromes examined so far (36) suggest that dominant negative inhibition of RA signaling by PML-RARA plays a critical role in the pathogenesis of APL (25). However, RARA-deficient ($RARA^{-/-}$) mouse models revealed that the retinoid signal is dispensable for myeloid differentiation (11); therefore, how PML-RARA leads to APL requires a revision.

PML contains a characteristic triad of a RING finger, two B boxes (B1 and B2), and a coiled-coil motif, which participates in the formation of high-order multiprotein complexes (20). PML forms discrete "speckle" structures in the nucleus called PML oncogenic domains (PODs) (40). Although the biological functions of PODs remain unclear, they are disrupted by PML-RARA into "microspeckle" structures, which are a hallmark of APL (20). There are several isoforms of PML, which differ in their C termini as a result of alternative splicing (9). Although PML proteins have a number of pleiotropic functions, such as regulation of proliferation, apoptosis, or senescence, at least in vitro (20), little is known about the specific activities of each isoform. PML has been highlighted as a transcriptional coregulator, since it associates with several transcription factors and cofactors (40). Despite the ubiquitous expression of PML proteins and the variety of binding partners, PML-deficient ($PML^{-/-}$) mice do not display significant phenotypes, suggesting that they are not required for but rather modulate normal development. Interestingly, however, the terminal differentiation of granuloid and monocytoid cell lineages is impaired in $PML^{-/-}$ mice (32), but how this occurs remains to be elucidated.

Granulopoiesis is a tightly regulated developmental process that begins with the commitment of myeloid precursors followed by their terminal differentiation, a process that requires cooperative or stepwise actions of lineage-specific transcription factors (6). PU.1 is expressed exclusively in hematopoietic cells (13), and it binds to a purine-rich DNA sequence containing the 5′-GGAA/T-3′ core motif. Although the targeted disruption of the *PU.1* gene can cause multiple hematopoietic aberrations, it invariably causes a defect in the terminal differentiation of myeloid cells (5, 19, 22, 27). PU.1 associates with the p300/CREB-binding protein (CBP) coactivator, at least in vitro (33); however, its essential protein-protein interactions during granulocytic terminal differentiation remain to be explored. PU.1 regulates many myeloid cell-specific genes, including cytokine receptors for granulocyte, granulocyte-macrophage, and macrophage colony-stimulating factor, but the transcriptional cascade that underlies the cell-autonomous effects of PU.1 remains to be elucidated.

CCAAT/enhancer-binding protein ε (C/EBPε) is expressed exclusively in granuloid cells and is essential for the terminal

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differentiation of committed granulocyte progenitors (17, 34a). C/EBPε is thought to be one critical target of PML-RARA. This is strongly supported by the following observations: (i) C/EBPε is directly regulated by RARA (23); (ii) PML-RARA inhibits the expression of C/EBPε, whereas ATRA restores it (23); (iii) the introduction of C/EBPε into APL cells can mimic the ability of ATRA to drive granulocyte differentiation in vitro and repress the leukemic phenotype of APL in vivo (29); and (iv) APL cells lack secondary granules, which is the prominent characteristic of APL cells and is consistent with a deregulation of C/EBPε expression (7, 34a).

To clarify the role of PML-RARA in the pathogenesis of APL, especially how it causes the arrest of granulocytic differentiation, we focused on the biological properties of PML-RARA and how it perturbs PML function. We show for the first time that the impaired granulopoiesis in *PML*$^{-/-}$ mice is associated with the reduced expression of C/EBPε. In addition, PU.1 directly regulates C/EBPε expression, and PML modulates it by promoting the formation of a PU.1/p300 complex in an isoform-specific manner. Finally, we show that PML-RARA can block granulopoiesis by the direct transrepression of a PU.1/PML/p300 ternary complex and propose a model for how PML-RARA acts as a dominant-negative inhibitor of PML-induced transcription. These results should provide a new insight into how PML-RARA causes leukemia and should help identify new molecular targets for the treatment of APL.

MATERIALS AND METHODS

Mice and cell lines. PML-deficient mice were generated as described previously (32). Mice over 40 weeks of age were analyzed. All animals were maintained under specific-pathogen-free, temperature-controlled conditions throughout this study, in accordance with institutional guidelines. Written approval for all animal experiments was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

Interleukin-3-dependent myeloid L-G myeloblasts (16) and BOSC23, NIH 3T3, and HeLa cells were obtained from the Japanese Cancer Research Bank (Osaka, Japan).

Flow cytometric analysis. Bone marrow (BM) and peripheral blood cells were prepared by lysing erythrocytes in ammonium chloride buffer. In general, one million cells were incubated on ice for 45 min with the appropriate staining reagents according to standard methods. The reagents used in this study were as follows: peridinin chlorophyll protein-cyanin 5.5-conjugated streptavidin, anti-Mac-1–fluorescein isothiocyanate (M1/70-fluorescein isothiocyanate), anti-c-Kit–allophycocyanin (2B8-allophycocyanin), and anti-Gr-1–biotin (RB6-8C5-biotin). All of the reagents were purchased from Pharmingen (La Jolla, CA). Flow cytometry was performed by using a FACSCalibur apparatus (Becton Dickenson), and the results were analyzed using CELLQUEST software (Becton Dickenson).

Expression vectors. Human cDNAs encoding FLAG- or hemagglutinin (HA)-tagged PML isoforms I, II, III, IV, V, and VI were cloned into pLNCX and pLPCX retroviral mammalian expression vectors as described elsewhere previously (21). The cDNAs for PU.1, PML-RARA, PLZF-RARA, RAR, and retinoid X receptor were kindly provided by Francoise Moreau-Gachelin, Akira Kakizuka, Zu Chen, and Pierre Chambon, respectively. The deletion mutants for PU.1 and PML were constructed by PCR-mediated methods (see the supplemental material). The cDNAs for FLAG-tagged PU.1 and its deletion mutants were cloned into the metallothionein promoter-driven expression vector pMT-CB6+. The PML-RARA cDNA was cloned into the pcDNA3.1/His vector (Invitrogen, Carlsbad, CA) for Xpress tagging. HA-tagged PML-RARA cDNA was also cloned into the puromycin-resistant vector pMT-CB6+puro. HA- or FLAG-tagged p300 expression vectors were described previously (21).

Construction of L-G myeloblast clones. L-G cells (1×10^7) were transfected with 10 μg of PvuI-linearized plasmid pMT-CB6+/PU.1 (FLAG tagged) or its deletion derivatives by electroporation (960 μF, 0.35 kV). Stable clones were selected by the treatment of the cells with 1 mg/ml of G418. The expression of PU.1 was induced with 100 μM of $ZnSO_4$ and verified by Western blotting. Retroviruses were prepared from BOSC23 cells transfected with vector pLPCX encoding HA-tagged PML isoforms I to VI or C-terminal deletion mutants of PML IV, and bulk populations were selected by treatment with 0.6 μg/ml of puromycin.

Antibodies. The following antibodies were used in this study: anti-PU.1 (T-21 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (M2 mouse monoclonal; Sigma, St. Louis, MO), anti-p300 (NM11 mouse monoclonal; BD Bioscience, San Diego, CA;), anti-HA (3F10 rat monoclonal; Roche Diagnostics, Mannheim, Germany), anti-C/EBPε (C-22 rabbit polyclonal; Santa Cruz Biotechnology), anti-TFIIB (C-18 rabbit polyclonal; Santa Cruz Biotechnology), and anti-PML (PML001 rabbit polyclonal and 1B4 mouse monoclonal [MBL, Nagoya, Japan] and H-238 rabbit polyclonal [Santa Cruz Biotechnology]).

Immunoprecipitation and Western blotting. Detailed procedures for sample preparation, immunoprecipitation, and Western blotting were described elsewhere previously (21).

Immunostaining. Indirect immunofluorescence was performed as previously described (21).

EMSA. An electrophoretic mobility shift assay (EMSA) was performed according to standard procedures (see the supplemental materials for details). For supershift assays, an anti-PU.1 polyclonal antibody (Santa Cruz Biotechnology) was used.

ChIP. Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's instructions (UBI, Lake Placid, NY) by using the following primers: 5′-CCCATGAGTACCTATATGCTCA-3′ and 5′-CTCAAATCTGGC CTCCGTCACTG-3′ for region 1, 5′-AAGGCTTACATCTCTCCCTCTG-3′ and 5′-CTGTCACCCACTCCTGTGTG-3′ for region 2, and 5′-CACACGATT GTTTAGAGGTAGAAC-3′ and 5′-GAGACTTTAAGAAGCCCGTAATC-3′ for region 3.

Luciferase reporter assay. The *C/EBPε* promoter region was amplified by genomic PCR and cloned into the pGL3-Basic vector (Promega, Madison, WI) as described previously (34). The putative PU.1 binding sites were mutated by site-directed mutagenesis according to standard procedures (see the supplemental material for details). The cells were transfected with the aid of Effectene transfection reagent according to the manufacturer's instructions (QIAGEN, Valencia, CA). After 36 h, luciferase activity was determined using a Dual Luciferase assay system (Promega) according to the manufacturer's instructions. Values were normalized by the luciferase activity of a cotransfected *Renilla* luciferase-expressing vector (pRL-CMV).

RT-PCR. RNA was isolated according to standard protocols and reverse transcribed with random primer using Superscript II (Invitrogen) according to the manufacturer's instructions. For quantitative reverse transcription (RT)-PCR, the following sets of primers and internal fluorescence probes were purchased from Roche Diagnostics (Indianapolis, IN); mouse *C/EBPε* (catalog no. 04688970001), mouse *GAPDH* (catalog no. 04689089001), and mouse *PML* (catalog no. 04688996001). PCRs were performed using an ABI PRISM 7500 Fast Real-Time RCR system (Perkin-Elmer, Foster City, CA) using TaqMan Universal PCR Master Mix containing specific primers (1.2 μM) and a specific probe (0.1 μM). For conventional RT-PCR, the following sets of primers were used: 5′-GACTACAAAGACGATGACGAC-3′ (forward) and 5′-CAGTAATGGTC GCTATGGCTC-3′ (reverse) for FLAG-tagged human *PU.1* and 5′-CTTCACC ACCATGGAGAAGG-3′ (forward) and 5′-GGCATGGACTGTGGTCATGA G-3′ (reverse) for *GAPDH*.

RESULTS

Impaired granulopoiesis and reduced C/EBPε expression in PML-deficient mice. Flow cytometry analysis of sex-matched littermates revealed that circulating Gr-1^{hi} Mac-1^{+} mature granulocytes were reduced in peripheral blood from *PML*$^{-/-}$ mice (Fig. 1A), as previously described (32). On the other hand, the increase of immature granulocytes in the BM of *PML*$^{-/-}$ mice was demonstrated by a fourfold increase in Gr-1^{+} c-Kit^{+} cells (Fig. 1B). These results suggest that the terminal maturation of granulocytes is impaired in *PML*$^{-/-}$ mice. To investigate the role of PML in normal granulopoiesis, we examined the expression levels of several transcription factors that are supposed to be essential for the process. Western blotting analysis of BM mononuclear cells revealed that PML expression was reduced in proportion to the *PML* genotype.