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Impaired repressor activity and biological functions of PU.1 in MEL cells induced by mutations in the acetylation motifs within the ETS domain

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Abstract

PU.1, a hematopoietic Ets transcription factor, is required for development of the lymphoid and myeloid lineages. We have previously shown that PU.1 functions as both a transcriptional activator and repressor through complex formation with CBP/p300 and HDAC1/mSin3A/MeCP2, respectively. To determine whether modification of PU.1 is responsible for switching its association between co-activators and co-repressors, we examined whether acetylation regulates the physical and functional activities of PU.1. PU.1 was acetylated in vivo and its repressor activity was reduced when the putative acetylation motifs in the Ets domain were mutated. The mutant cooperated with CBP similar to wild type PU.1, but insufficiently with GATA-1 and mSin3A. Whereas overexpression of wild type PU.1 induced differentiation block, growth inhibition, and apoptotic cell death in MEL erythroleukemia cells as we reported previously, overexpression of the mutant-acetylation motif PU.1 did not. Taken together, our data suggest that acetylation might regulate the biological functions of PU.1 in erythroid cells.
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Keywords: PU.1; Acetylation; Transcriptional activity; Differentiation; Growth; Apoptosis; Erythroid cells

PU.1 is a member of the Ets transcription factor family, which is characterized by the presence of a DNA-binding domain that recognizes a core DNA element containing the 5′-GGAA/T-3′ motif [1]. Its expression is restricted to hematopoietic cells, predominantly B cells and macrophages. Knockout mouse studies have shown that PU.1 deficiency results in a lack of normal B cells and macrophages, disrupted granulopoiesis, and aberrant T lymphopoiesis [2]. This suggests that PU.1 may regulate some genes necessary for the development of both the lymphoid and myeloid lineages [3,4].

The *PU.1* gene is identical to the *Spi-1* (*Sfpi*-1) gene, the expression of which is deregulated as a consequence of pro-viral integration of spleen focus-forming virus (SFFV) in most Friend virus-induced murine erythleukemia (MEL) cells [5]. In our previous study, we showed that overexpression of PU.1 in MEL cells inhibited cell growth and differentiation, and induced apoptosis [6].

PU.1 has been shown to interact with various transcription factors including NF-EM5/PIP, c-Jun, and GATA-1 [7–9]. Importantly, in some cases, PU.1 regulates gene transcription by interaction with these regulatory factors through phosphorylation-mediated modifications [10–12]. However, it is not clear whether modifications other than phosphorylation are concerned with such interactions between PU.1 and other transcription factors or co-factors. CBP/p300 acetylates a number of nuclear proteins besides histones and plays a role as co-activator of various types of transcription

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factors [13]. The target proteins include transcription factors like GATA-1, TAL1/SCL, EKLF, YY1, and p53 [14–20]. Analogous to phosphorylation, acetylation appears to modulate diverse protein functions which subsequently result in altered gene expression. We previously demonstrated that PU.1 functions both as an activator and as a repressor through interactions with CBP/p300 [21] and HDAC/mSin3A/MeCP2, respectively [22,23]. In this study, we investigated the effects of mutations in the acetylation motifs of PU.1 on the physiological and functional activity of the factor. We found that PU.1 was acetylated in vivo and its transcriptional repressor activity was impaired by mutations within the acetylation motifs. Mutant PU.1 interacted with mSin3A and GATA-1 less avidly than did wild type PU.1, while it significantly bound CBP. Furthermore, PU.1 having mutations in its acetylation motifs could not induce differentiation block, growth inhibition, and apoptosis in MEL cells, while wild type PU.1 could induce all these effects. Thus, these results suggest that acetylation of PU.1 might regulate PU.1 activity in erythroid cells through dissociation of mSin3A and/or GATA-1.

Materials and methods

Cell culture. 293T, COS1, and Saos2 cells were maintained in DMEM supplemented with 10% fetal bovine serum. A zinc-inducible plasmid containing wild type (WT) PU.1 or PU.1 having mutations in the putative acetylation motifs (mut-AB), or an empty vector was introduced into MELB8/3 cells by electroporation as described [6]. Several independent clones were established by G418 (Wako) selection. Clones were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum. Cells were serially maintained in 5% CO_2 at 37 °C.

Plasmid constructions. A FLAG-tagged PU.1 expression vector (FLAG-PU.1) and an elongation factor promoter-mediated PU.1 expression vector (pEF-PU.1) have been described previously [21,22]. Several expression vector constructs containing lysine to arginine substitutions in the PU.1 acetylation motifs (mut-A, mut-B, and mut-AB) were constructed from FLAG-PU.1 using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Following mutagenesis, the variously mutated full-length PU.1 variants were separately inserted into a pEF expression vector and the metallothionein promoter-derived zinc-inducible expression plasmid. The pEF and pMT expression vectors were kindly donated by Dr. K. Nagata, Osaka University and Dr. M. Obinata, Tohoku University, Japan, respectively. A plasmid encoding GST-mSin3A272–680 was also constructed by inserting the respective mSin3A sequences from amino acid residues 272 to 680 into pGEX-4T-2. GST-CBP1890 was as described previously [21,22]. GST-GATA-1 was kindly provided by Dr. T. Nakano, Osaka University, Japan.

In vivo acetylation and immunoprecipitation. Ten micrograms of FLAG-PU.1 or empty vector (pCMV–Tag2; STRATAGENE) was transfected into COS-1 cells. After 30 h, cells were exposed to 1 mCi of sodium [^{3}H]acetate per milliliter in the presence of 50 nM Trichostatin A for 90 min. Cells were lysed in TNE buffer (10 mM Tris–HCl [pH 7.8], 150 mM NaCl, 0.5% NP40, and 1 mM EDTA) supplemented with 5% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates were immunoprecipitated using anti-FLAG antibody (M2; Stratagene) and the immunoprecipitated complexes were then resolved by Nu-PAGE (Invitrogen) [14]. Following Nu-PAGE analysis, the specific bands were revealed by FLA 2000 Image Analyzer (Fuji Film) and Western blotting using anti-PU.1 antibody (T-21; Santa-Cruz).

Luciferase assays. Reporter plasmids of either pM-CSF-R Luc [4] or myc (*Ava*I–*Sac*I) Luc [22] (0.2 μg) were transfected into 293T or Saos2 cells together with the pEF expression vector for PU.1 (50 ng–0.2 μg) using Lipofectamine Plus (Gibco). Cells were harvested 24 h later and luciferase assays were performed as described previously [22].

GST pull-down assay. GST fusion proteins were prepared as previously described [21]. The pEF expression vector for PU.1 was transiently transfected into 293T cells by Lipofectamine Plus (Gibco) and the cells were lysed with TNE buffer supplemented with the protease inhibitors. GST fusion proteins were immobilized onto glutathione–Sepharose 4B (Pharmacia), washed in phosphate-buffered saline (PBS), and incubated with cell lysates for 3–4 h. Bound proteins were washed three times with TNE buffer, separated in Nu-PAGE (Invitrogen), and detected by Western blot analysis using anti-PU.1 antibody (T-21; SantaCruz) as described earlier.

Cell growth and cell death analysis. Cells ($1 - 2 \times 10^6$) were plated in 10 ml culture medium containing 10% serum with or without 100 μM $ZnCl_2$ or 100 μM $ZnCl_2$ and 1.5% DMSO, a differentiation agent. The total number of cells was counted 1, 3, and 4 days later. The number of dead cells was counted by trypan blue dye exclusion assay after 3 days.

RT-PCR. The transfectants were cultured under conditions with or without 100 μM $ZnCl_2$ and/or 1.5% DMSO. We isolated RNA 3 days after initiating culture and subsequently performed RT-PCR analysis [24]. The primers used were as described previously [24].

Results

In vivo acetylation of PU.1

We have previously shown that CBP/p300 and mSin3A/HDAC1 interact with PU.1 to regulate its transcription activity [21,22]. It has been shown that CBP/p300 possesses intrinsic acetyltransferase activity and HDAC1 shows deacetylase activity. Analysis of the PU.1 amino acid sequence reveals that PU.1 contains several lysine residues within the Ets domains identified as potential substrates for acetylation. To determine whether acetylation of PU.1 indeed occurs in vivo, we investigated acetylation of PU.1 in transient transfection experiments as described under Materials and methods. The autoradiograph in Fig. 1A demonstrated that a ^{3}H-labeled band was detected in COS1 cells transfected with the PU.1 expression vector but not with the empty vector. Western blot analysis using anti-PU.1 antibody revealed that the detected band was ^{3}H-labeled PU.1. These results suggest that acetylation of PU.1 occurs in vivo.

The repressor activity of PU.1 is impaired by mutations in the acetylation motifs

To elucidate the effect of PU.1 acetylation on transcriptional activity, we performed luciferase assay using a PU.1 expression vector containing the lysine to

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