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Requirement of the coiled-coil domain of PML-RARα oncoprotein for localization, sumoylation, and inhibition of monocyte differentiation

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Abstract

Homo-oligomerization via a coiled-coil (C-C) domain has been shown to be necessary for the promyelocytic leukemia (PML)-retinoic acid receptor-α (RARα) fusion protein to acquire oncogenic potential in acute promyelocytic leukemia. We show here that PML(ΔC-C)-RARα, which contains a deletion in its C-C domain, is neither localized as characteristic microspeckles nor modified by small ubiquitin-like modifiers (SUMO). The absence of sumoylation of the ΔC-C mutant was due to the lack of binding to Ubc9, a SUMO conjugation enzyme. The integrity of RING finger domain was also needed for both sumoylation and microspeckle formation. In GAL4-DNA tethering assays, the ΔC-C mutant completely lost the inhibitory effect on retinoic acid (RA)-mediated transactivation. Furthermore, the expression of CD14 in U937 cells expressing the ΔC-C mutant in response to vitamin D3 was markedly higher than in cells expressing PML-RARα. However, the RA-mediated induction of C/EBPβ in cells expressing the ΔC-C mutant was comparable to that of control cells. Thus, our results suggest that the C-C domain-associated functions of sumoylation, localization as microspeckles, and the inhibition of monocyte differentiation all contribute to the oncogenic activity of PML-RARα.
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Keywords: PML; PML-RARα; SUMO modification; Coiled-coil domain; Oligomerization; Localization; Oncogenesis; Differentiation; Transactivation; C/EBPβ

The fusion of the promyelocytic leukemia (PML) gene to the retinoid acid receptor-α (RARα) gene in t(15;17) chromosomal translocation generates the chimeric PML-RARα gene, which is a characteristic of acute promyelocytic leukemia (APL) [1–3]. Several studies using animal models support the idea that expression of PML-RARα protein leads to the inhibition of cell differentiation at the PML stage of bone marrow development [4–6]. Four other fusion partners of the RARα gene have been reported in APL, namely, promyelocytic leukemia zinc finger (PLZF), nucleophosmin (NPM), nuclear mitotic apparatus (NuMA), and stat5b genes (for review, see [7]).

The fact that all fusions occur through the RARα gene suggests that APL is generated by the ablation of the RARα signaling pathway, in which RARα heterodimerizes with retinoid X receptor (RXR). In the absence of retinoic acid (RA), this heterodimer recruits a co-repressor complex containing SMRT or N-CoR, mSin3, and histone deacetylase (HDAC), which leads to transcriptional repression [8–10]. However, in the presence of RA, this co-repressor complex is dissociated and a co-activator complex is recruited, which results in transcriptional activation (for review, see [11]).

PML-RARα and PLZF-RARα are the most well-known fusion proteins that cause APL. Both fusion

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proteins can bind to retinoic acid (RA)-responsive elements (RAREs) as homodimers or as multimeric complexes containing retinoid X receptors (RXRs). However, their responses to RA differ. At low RA concentrations, both fusion proteins inhibit RA-mediated transactivation of reporter gene and cell differentiation. However, at high RA concentrations, this inhibitory effect of PML-RARα, but not that of PLZF-RARα, is relieved (for reviews, see [12,13]).

PML protein contains the so-called RBCC motif, which consists of a C3HC4 zinc finger (RING finger), cystein/histidine-rich regions (B-boxes), and a coiled-coil (C-C) domain [14]. In normal cells PML is localized in subnuclear structures known as PML oncogenic domains (PODs) or PML nuclear bodies (NBs). PML is covalently modified by small ubiquitin-like modifiers (SUMO) [15]. Recent studies have demonstrated that post-translational SUMO modification (sumoylation) is required for the formation of mature PODs and for the recruitment of other cellular proteins such as Daxx, Sp100, and PA28 into PODs [16–19]. In APL cells, PML-RARα expression causes a redistribution of POD-associated proteins into microspeckles (micropunctate forms) [20–22]. However, exposure to RA or arsenic trioxide (As_2O_3) results in the degradation of PML-RARα and leads to the restoration of normal PODs and a reinstatement of the ability of cells to differentiate. Like PML, PML-RARα is also modified by SUMO [23]. A recent study showed that As_2O_3 treatment induces both sumoylation and the proteasome-dependent degradation of PML and PML-RARα [17].

The C-C domain of PML-RARα mediates protein oligomerization, the efficient binding to co-repressors, and has some inhibitory effect on RA responsiveness [24,25]. Moreover, the ability of PML-RARα to abrogate RB or MAD-mediated transcription repression was also found to require the C-C domain [26,27]. Recently, PML-RARα was shown to induce the expression of C/EBPβ, a C/EBP family member, after RA treatment in APL cells and in monocytic cells expressing PML-RARα, which suggests that C/EBPβ is a downstream target gene of PML-RARα in the RA-induced differentiation of APL cells [28]. Despite accumulating evidence that the C-C domain of PML-RARα is involved in several of the functions of this fusion protein, the roles of this domain with respect to intranuclear targeting, sumoylation, and the regulation of transcription have not been fully addressed. In the present study, we show that the C-C domain of PML-RARα is required for the characteristic localization of the fusion protein as microspeckles, its covalent modification by SUMO, and for the inhibitory effect of PML-RARα on RA-mediated transactivation. Moreover, we show that this domain is also involved in blocking monocytic cell differentiation, thus demonstrating that these C-C domain-associated functions may all contribute to the oncogenic activity of PML-RARα. However, these C-C domain-associated functions were not required for the RA-mediated induction of C/EBPβ.

Materials and methods

Cell culture and virus infection. Vero, HeLa, and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). H29D cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine (Gibco), 50 U/ml penicillin and streptomycin (Gibco), and 2 mg/ml tetracycline. U937 cells were grown in RPMI medium supplemented with 10% FCS. To maintain U937 cell lines expressing the PML-RARα fusion proteins, 2 mg/ml puromycin (Sigma) and 0.3 mg/ml G418 (Gibco) were added to the medium.

Transient DNA transfection. For indirect immunofluorescence assay (IFA), Vero cells were seeded into 2-well slide chambers and DNA transfection was carried out using FuGene 6 reagents (Roche). For immunoblot analysis, 293T cells were seeded into 6-well plates and DNA mixtures were introduced into subconfluent cells using the *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline (BBS) version of the calcium phosphate procedure described previously [29]. For luciferase (LUC) reporter assays, HeLa cells were seeded into 12-well plates and DNA transfection was carried out using Lipofectamine 2000 reagents (Invitrogen).

Plasmid construction. Expression plasmid (pUS112) encoding HA-tagged PML VI, a 560 amino acid version of PML [14], was previously described [30]. Plasmids containing the cDNAs of PML-RARα and PLZF-RARα were provided by Jae-Hoon Lee (Baylor University, Texas). Expression plasmids for HA-PML-RARα (pJHA387) or HA-PLZF-RARα (pJHA389) were generated on a pSG5 [31] background using Gateway technology (Invitrogen). Plasmid for HA-PML(ΔC-C)-RARα (pJHA409) with a deleted coiled-coil (C-C) domain from amino acid positions 229 to 360 was generated using a polymerase chain reaction (PCR)-based procedure. Plasmid for HA-PML(CP>SR)-RARα (pJHA457) containing substitutions of Cys and Pro at positions 88 and 89 with Ser and Arg, respectively, within the RING finger domain was constructed using the Stratagene QuickChange site-directed mutagenesis procedure. Mammalian expression plasmids for GAL4-DNA binding (DB) domain fusions GAL4-DB/PML VI (pUS130), GAL4-DB/PML-RARα (pJHA445), GAL4-DB/PML(ΔC-C)-RARα (pJHA446), GAL4-DB/PML(CP>SR)-RARα (pJHA459) or GAL4-DB/PLZF-RARα (pJHA447) were generated on a pSV2-GAL4 background using Gateway technology.

Yeast plasmids expressing GAL4-DB/PML-RARα fusion (pJHA482), GAL4-DB/PML(ΔC-C)-RARα (pJHA484), GAL4-DB/PML(CP>SR)-RARα (pJHA483), or GAL4-activation (A) domain/PML-RARα fusion (pJHA485) were generated on pAS1-CYH2 (for GAL4-BD fusions) or pACTII (for GAL4-A fusions) backgrounds [32] using Gateway technology. Yeast plasmids for GAL4-A alone (pACTII) and GAL4-A/Ubc9 were previously described [32]. A reporter plasmid containing $(GAL4)_5$/TK-LUC was previously described [33].

Generation of U937 cell lines. U937 cell lines that constitutively expressed PML-RARα, PML(ΔC-C)-RARα, or PLZF-RARα were generated by infecting cells with recombinant retroviruses expressing the fusion proteins. The MFG retroviral vector constructs [34] encoding PML-RARα, PML(ΔC-C)-RARα, or PLZF-RARα were generated by PCR cloning. After transfecting H29D cells with the MFG retroviral vectors, viral supernatants were harvested at 72 h and passed through a 0.45 mM filter. Supernatants were added to 4 μg/ml Polybrene (Sigma) and overlaid on U937 cells on 6-well plates (5×10^5 cells/well). After incubation for 24 h, supernatants were replaced with normal media. The next day, cells were selected with 0.1μg/ml puromycin. Clones were isolated by dilution cloning after one week.

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