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Biochemical and Biophysical Research Communications 365 (2008) 258–265
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Coiled-coil domain of PML is essential for the aberrant dynamics of PML-RARα, resulting in sequestration and decreased mobility of SMRT

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Received 21 October 2007
Available online 6 November 2007

Abstract

Promyelocytic leukemia–retinoic acid receptor α (PML-RARα) is the most frequent RARα fusion protein in acute promyelocytic leukemia (APL). Our previous study has demonstrated that, compared with RARα, PML-RARα had reduced intranuclear mobility accompanied with mislocalization. To understand the molecular basis for the altered dynamics of PML-RARα fusion protein, we performed FRAP analysis at a single cell level. Results indicated that three known sumoylation site mutated PML-RARα had same intracellular localization and reduced mobility as wild-type counterpart. The coiled-coil domain of PML is responsible for the aberrant dynamics of PML-RARα. In addition, we revealed that co-repressor SMRT co-localized with PML-RARα, resulting in the immobilization of SMRT while ATRA treatment eliminated their association and reversed the immobile effect of SMRT. Furthermore, co-activator CBP, co-localized with PML-RARα in an ATRA-independent way, was demonstrated as a high dynamic intranuclear molecule. These results would shed new insights for the molecular mechanisms of PML-RARα-associated leukemogenesis.

Keywords: PML-RARα; Coiled-coil domain; SMRT; CBP; FRAP

Acute promyelocytic leukemia (APL) is characterized by a terminal differentiation block of myeloid development and a specific reciprocal chromosomal translocation, involving the promyelocytic leukemia (PML) gene at 15q22 and the retinoic acid receptor α (RARα) gene at 17q21, which was regarded as a diagnostic hallmark of APL [1,2]. This frequently occurred fusion gene was translated into PML-RARα fusion protein found to be responsible for the cellular transformation and the initiation of APL [3]. Four rare variant translocations occurred in the remaining cases of APL, each disrupting the RARα locus on chromosome 17 [1].

The fact that the RARα gene is involved in all of chromosomal translocations in APL suggests that APL is generated by the disruption of the RARα signaling pathway, in which RARα heterodimerizes with retinoid X receptor (RXRα). The transcriptional regulation by RARα/RXRα heterodimer involves the exchange of co-repressor complexes, such as SMRT/NCoR and HDAC3, and co-activator complexes, such as SRC-1, SRC-3, and CBP, which are dependent on hormone binding status of the receptor [1,2]. PML-RARα is acted as a potent repressor for silencing RARα targeting genes through forming PML-RARα homo-oligomers, which tightly bound to co-repressor SMRT [1,2,4]. The transcriptional switch from repression to activation is achieved by the pharmacological concentration of all-trans retinoic acid (ATRA) treatment, resulting in a completely terminal differentiation of leukemic cells in vivo and ex vivo [3].

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.184

Sumoylation is a post-translational modification that covalently attaches an ubiquitin-like peptide, SUMO (small ubiquitin-related modifier), to specific lysine residues in target proteins, which regulates a wide range of cellular processes, including gene transcription, chromatin organization, protein–protein interactions, and subcellular localization. PML sumoylation is required for the recruitment of its partner proteins and the formation of PML nuclear bodys (NBs), but not for its nuclear matrix targeting [5,6]. The normal PML NB structures within the nucleus are disrupted when expression of PML-RARα resulting in a characterized microspeckled structure, usually considered to interfere with the normal function of PML. A specific sumoylation site K160 in the PML-RARα was essential to the leukemic transformation of primary progenitors ex vivo and was required for the arsenic trioxide induced PML-RARα degradation [7,8].

Although much has been learned regarding the pathogenetic and therapeutic mechanisms of APL in the past two decades, more remains to be discovered regarding how PML-RARα really interrupts the normal function of RARα or PML at a single cell level. Our previous data showed that intranuclear mobilities of RARα-fusion and AML1-fusion proteins were reduced accompanied with altered cellular localization compared to wild-type RARα or AML1 [9,10], implying that the aberrant dynamics might be a more common and complicated properties of fusion proteins in leukemia. In present study, we used fluorescence recovery after photobleaching (FRAP) assay to study the roles of sumoylation and a homo-oligomerization domain of PML-RARα contributed to the aberrant intracellular dynamics of this fusion protein and found that PML-RARα had an effect on the intranuclear localization and mobility of its co-regulators SMRT and CBP.

Materials and methods

Plasmid construction. The cyan fluorescent protein (CFP)-tagged PML-RARα, yellow fluorescent protein (YFP)-SMRT, and YFP-CBP have been reported previously [10–12]. The green fluorescent protein (GFP)-tagged PML-RARα was made by a swap of PML-RARα cDNA into pEGFP-C1 vector (Clontech) from CFP-PML-RARα. The mutants of GFP-PML-RARα including three PML-RARα sumoylation site mutants—PML-RARα (K65R), PML-RARα (K160R), PML-RARα (K490R)—and PML-RARα (ΔCC), in which the coiled-coil domain of PML is deleted, were generated by PCR approach as previous description [10]. All of constructs were confirmed by DNA sequencing and Western blot analysis.

Cell culture and reagents. HeLa and 293T cells were maintained in Opti-MEMI medium (Invitrogen) with 4% FBS and in Dulbecco Modified Eagle Medium (DMEM) (Invitrogen) with 10% FBS, respectively [9]. All-trans retinoic acid (ATRA) was purchased from Sigma (St. Louis, MO).

Gel-shift assay. The whole cell extracts (WCE) from 293T cells transiently transfected with GFP-PML-RARα, GFP-PML-RARα (K65R), GFP-PML-RARα (K160R), GFP-PML-RARα (K490R), GFP-PML-RARα (ΔCC), and RXRα expression vectors were incubated with radiolabeled DR5G in binding buffer (20 mM Hepes, pH 7.9, 0.5 mM EDTA, 2 mM DTT, 10% glycerol, 50 mM KCl, and 1 μg of poly dI-dC) for 30 min at 4 °C and subsequently for 30 min at room temperature. Protein–DNA complexes were run on 5% polyacrylamide gels equilibrated in 0.25× TBE and subsequently analyzed by PhosphorImager software.

Fluorescence microscopy and fluorescence recovery after photobleaching (FRAP). The fluorescence-tagged constructs were transfected into HeLa cells sit onto poly-D-lysine-coated coverslips in 24-wells plate using GeneJuice transfection reagent (Novegen) [9,10]. After 36–48 h transfection, cells were processed for fluorescence microscopy analysis using Zeiss LSM 510 confocal microscopes as described previously [9,10,12]. FRAP analysis was performed on Zeiss LSM 510 confocal microscopes using the laser set at a wavelength of 488 nm for GFP or wavelength of 510 nm for YFP at a maximum power for 50 or 30 iterations of a box representing ~20% of the nuclear volume, as indicated in our previous studies [9,10]. Fluorescence intensities of regions of interest (ROI) were obtained using LSM510 software, and data were exported to Excel (Microsoft) for analysis.

Data analysis. Unless indicated otherwise, data presented are the means ± SD; differences between means were assessed for significance using Student's *t*-test. Significance was assigned at $P < 0.05$.

Results

DNA binding activities of GFP-tagged PML-RARα and its mutant forms

We have reported that PML-RARα is immobilized within the nucleus using CFP-tagged PML-RARα construct [10]. To address whether the sumoylation of PML-RARα or the coiled-coil domain in PML-RARα contribute to the intracellular mislocalization and/or the reduced mobility of PML-RARα, we constructed several green fluorescent protein (GFP) tagged PML-RARα expression vectors, including GFP-PML-RARα(ΔCC), in which the coiled-coil domain of PML moiety was deleted (Fig. 1A), and three sumoylation mutants of GFP-PML-RARα—GFP-PML-RARα(K65R), GFP-PML-RARα(K160R), and GFP-PML-RARα(K490R), in which three known sumoylation sites located at lysine (L) 65, 160, and 490 residues within PML moiety were substituted by arginine (R), respectively [13]. The immunoblot analysis of whole cell extracts from transfected 293T cells showed that all of GFP tagged PML-RARα constructs encoded proteins of expected size (data not shown). By gel-shift assay, we demonstrated that all of GFP-tagged PML-RARα sumoylation mutants could bind to RARE as a homo-oligomer or as a hetero-oligomer with RXRα similar to GFP-PML-RARα and untagged counterpart (Fig. 1B) [3,10]. GFP-PML-RARα(ΔCC) showed a reduced DNA binding activity due to the loss of the homo-oligomerization domain within PML as previous reports, but rather it could efficiently bind to RARE as a hetero-oligomer with RXRα. Therefore, the addition of GFP tag did not significantly alter the DNA binding activity of these constructs.

Each single known sumoylation site is not required for the altered intranuclear distribution and reduced mobility of PML-RARα

We have shown that the intranuclear structure of PML-RARα oncogenic protein was distinct from both PML and RARα proteins and PML-RARα had slowing intranuclear mobility in comparison with wild-type RARα, which may

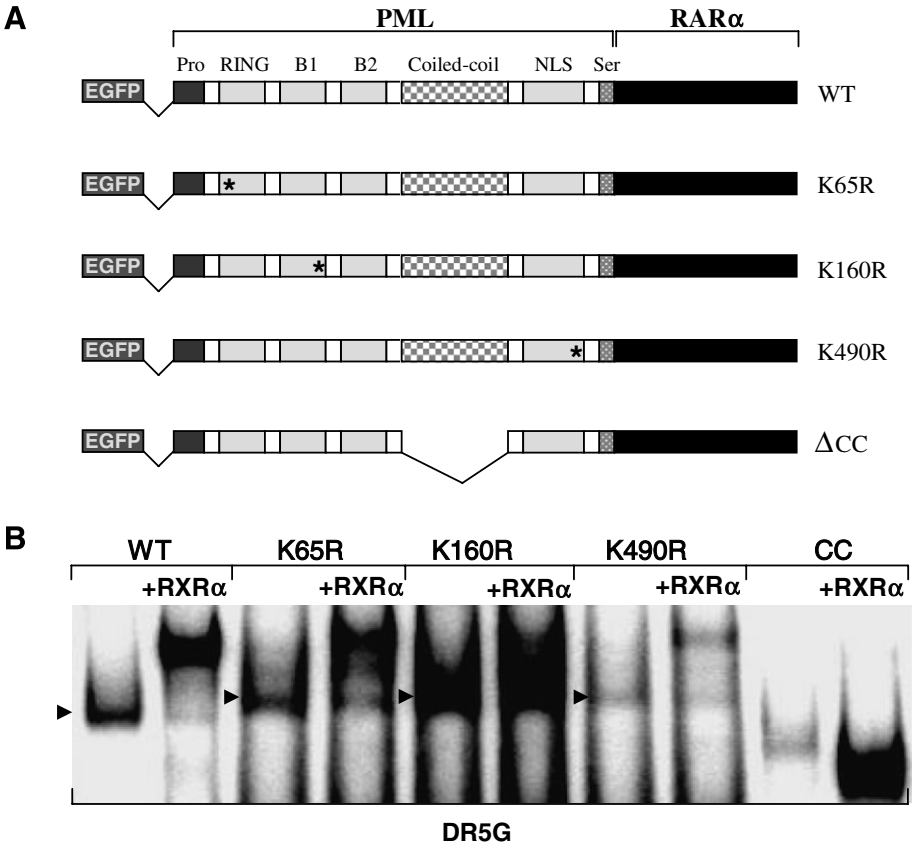

Fig. 1. DNA binding activities of GFP-tagged PML-RARα and its mutant constructs. (A) Schematic depiction of PML-RARα mutant constructs. * Represents three known sumoylated sites located within Ring, Box1, and NSL domain of PML moiety, respectively, in which lysine residue is substituted by arginine. WT indicated wild-type PML-RARα and ΔCC represented PML-RARα, in which the coiled-coil region is deleted. (B) Gel-shift assays were performed using radiolabeled DR5G and whole cell extracts of 293T cells transfected with the indicated GFP-tagged constructs with or without RXRα. The black arrowheads indicate the positions of GFP-PML-RARα or its mutant oligomer/RARE complexes.

be linked to the oncogenic function of PML-RARα [10]. To study whether the sumoylation of PML-RARα plays a role in its intranuclear mobility and intracellular localization, we performed FRAP analysis of GFP-tagged PML-RARα sumoylation mutants. The microspeckled distribution pattern within the nucleus was found in HeLa cells following transfection of these three sumoylation mutants of GFP-PML-RARα, similar to the wild-type counterpart (Fig. 2) [10]. Following photobleaching of HeLa cells transfected with GFP-tagged PML-RARα sumoylation mutants, the fluorescence recovery of these mutants were immobilized as wild-type PML-RARα [10]. Hence, each single known sumoylation site of PML-RARα does not contribute to the altered intranuclear distribution and immobilization of PML-RARα within nucleus.

The coiled-coil domain of PML-RARα is responsible for the slowing intranuclear mobility and intracellular localization of PML-RARα

Since the mis-localization and slowing intranuclear mobility of PML-RARα were not attributed to the single sumoylation site of PML, next we studied if the coiled-coil domain in PML-RARα fusion protein has impact on the oncogenic effects of PML-RARα. The coiled-coil (CC) domain within PML moiety has been shown to be necessary for homo-oligomerization of PML-RARα, specific microspeckled localization and inhibition of monocyte differentiation [14]. To examine the potential contribution of the coiled-coil domain in the immobilization of PML-RARα, we performed FRAP analysis of GFP-PML-RARα (ΔCC), in which the coiled-coil domain of PML-RARα was deleted. Indeed, GFP-PML-RARα (ΔCC) demonstrated a diffusion distribution pattern within nucleus similar to RARα, which was different from the microspeckled distribution pattern of the PML-RARα fusion protein, consistent with the previous report [14]. More interestingly, in contrast to GFP-PML-RARα, GFP-PML-RARα (ΔCC) showed a very fast recovery following photobleaching within nucleus, with fluorescence recovery half time ($t_{1/2}$) of 1.32 ± 0.58 s, which was very close to that of wild-type RARα (Table 1). Therefore, except for its contribution to other oncogenic activities of PML-RARα, the coiled-coil domain also plays a critical role in the intranuclear mobility reduction of PML-RARα (Table 1 and Fig. 2).

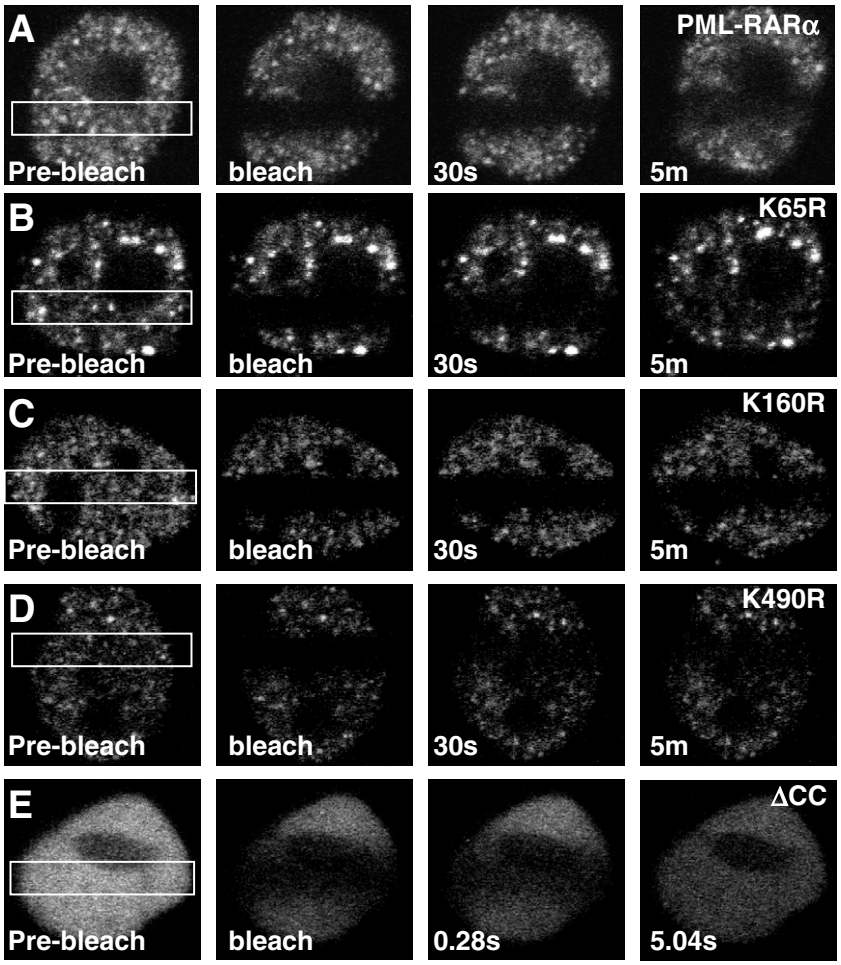

Fig. 2. Fluorescence recovery after photobleaching (FRAP) analysis of GFP-tagged PML-RARα and its mutants in live cells. The nuclei of HeLa cells transiently transfected with the GFP-PML-RARα (A), PML-RARα(K65R) (B), PML-RARα(K160R) (C), PML-RARα(K490R) (D), and PML-RARα(ΔCC) (E) were subjected to FRAP analysis. Images show a single z-section obtained before photobleaching (Pre-bleach), at the end of photobleaching (bleach), and at the indicated time points after photobleaching. The white rectangle represents the area bleached within the nucleus.

Table 1
Fluorescence recovery $t_{1/2}$ of GFP-PML-RARα, GFP-PML-RARα(K65R), GFP-PML-RARα(K160R), GFP-PML-RARα(K490R), and GFP-PML-RARα(ΔCC) in the absence of ATRA

No.	Construct	$t_{1/2}$ (−ATRA)
1	GFP-RARα	0.61 ± 0.18 s
2	GFP-PML-RARα	>5 min
3	GFP-PML-RARα (K65R)	>5 min
4	GFP-PML-RARα (K160R)	>5 min
5	GFP-PML-RARα (K490R)	>5 min
6	GFP-PML-RARα (ΔCC)	1.32 ± 0.58 s

$P = 0.0019$ for 1 versus 6.

Effect of PML-RARα on intranuclear localization and mobility of SMRT

The transcriptional co-repressors modulate gene expression by modifying the chromatin template and by making inhibitory contacts with the general transcriptional machinery. This effect may be dependent on the mobility of the co-repressors targeting their specific intracellular compartment. The co-repressor protein silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) was identified through its physical interaction with the nuclear receptors [2]. To examine the effects of oncogenic PML-RARα fusion protein on SMRT dynamics, we carried out FRAP analysis of the intranuclear mobility of YFP-SMRT in HeLa cells co-transfected YFP-SMRT with or without CFP-PML-RARα (Fig. 3). YFP-SMRT expression alone in HeLa cells demonstrated a distribution pattern with micro-speckles superimposed over a more diffuse nucleoplasmic localization as previously reported [15] and had fast intranuclear mobility with a recovery $t_{1/2}$ of 1.79 ± 0.52 and 1.73 ± 0.43 s in the absence and presence of ATRA, respectively ($P > 0.05$). Co-expression of cells with YFP-SMRT and CFP-PML-RARα not only resulted in YFP-SMRT co-localization with CFP-PML-RARα within nucleus in a microspeckled pattern in the absence of ATRA, but also led to a significantly reduced intranuclear mobility of YFP-SMRT (recovery $t_{1/2} > 5$ min; $P < 0.001$; Table 2 and Fig. 3). Following ATRA treatment (10^{-6} M for 2 h), YFP-SMRT dissociated from the location of CFP-PML-RARα examined by

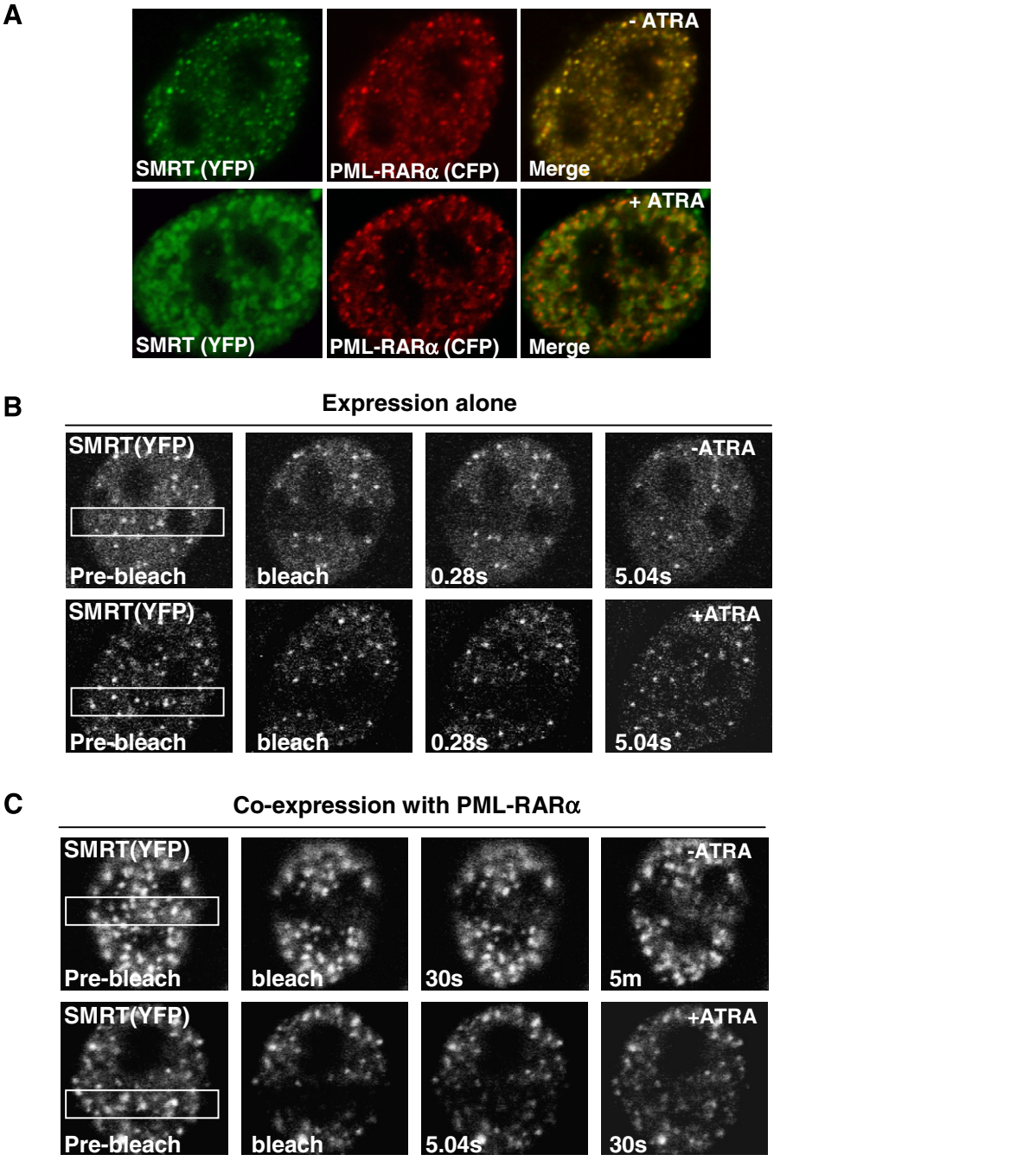

Fig. 3. FRAP analysis of YFP-SMRT co-expressed with or without CFP-PML-RARα in the absence and presence of ATRA. (A) Co-localization of YFP-SMRT and CFP-PML-RARα within the nucleus assessed by confocal fluorescence microscopy in the absence and presence of ATRA. After co-transfection of YFP-SMRT plus CFP-PML-RARα, HeLa cells were treated with or without 10^{-6} M ATRA for 2 h and imaged on the YFP channel (left panel) and CFP channel (middle panel). FRAP analysis of YFP-SMRT was performed on HeLa cells transfected with YFP-SMRT alone (B) or co-transfected with YFP-SMRT plus CFP-PML-RARα (C) in the absence or presence of 10^{-6} M ATRA for 2 h. Representative images show a single z-section obtained before photobleaching (Pre-bleach), at the end of photobleaching (bleach), and at the indicated time points after photobleaching. The white rectangle represents the area bleached within the nucleus.

Table 2
Fluorescence recovery $t_{1/2}$ of YFP-SMRT and YFP-CBP co-expressed with or without CFP-PML-RARα in the absence and presence of ATRA

No.	Constructs	$t_{1/2}$ (s) −ATRA	$t_{1/2}$ (s) +ATRA (10^{-6} M) for 2 h
1	YFP-SMRT	1.79 ± 0.52 s	1.73 ± 0.43 s ($P = 0.35$)
2	YFP-SMRT with CFP-PML-RARα	>5 min	3.54 ± 0.87 s ($P < 0.001$)
3	YFP-CBP	1.02 ± 0.35 s	1.06 ± 0.32 s ($P = 0.38$)
4	YFP-CBP with CFP-PML-RARα	0.88 ± 0.28 s	0.90 ± 0.26 s ($P = 0.44$)

$P < 0.001$ for 1 versus 2 in the absence of ATRA; $P = 0.003$ for 1 versus 2 in the presence of ATRA; $P > 0.05$ for 3 versus 4 in the absence and presence of ATRA.

confocal microscopy analysis, which efficiently reversed the reduced intranuclear mobility of YFP-SMRT, with the recovery $t_{1/2}$ of 3.54 ± 0.87 s ($P < 0.001$), closed to the mobility of YFP-SMRT transfection alone. These results indicate that PML-RARα can sequester SMRT into its address within nucleus and change its intranuclear mobility in an ATRA-dependent way.

CBP co-localized with PML-RARα in a ligand-independent manner does not alter the intranuclear mobility of CBP

PML-RARα could effectively sequester and co-localize with the co-repressor SMRT, leading to the reduced mobility of SMRT in a ligand-dependent way. Next, we examined whether PML-RARα could impact the intranuclear

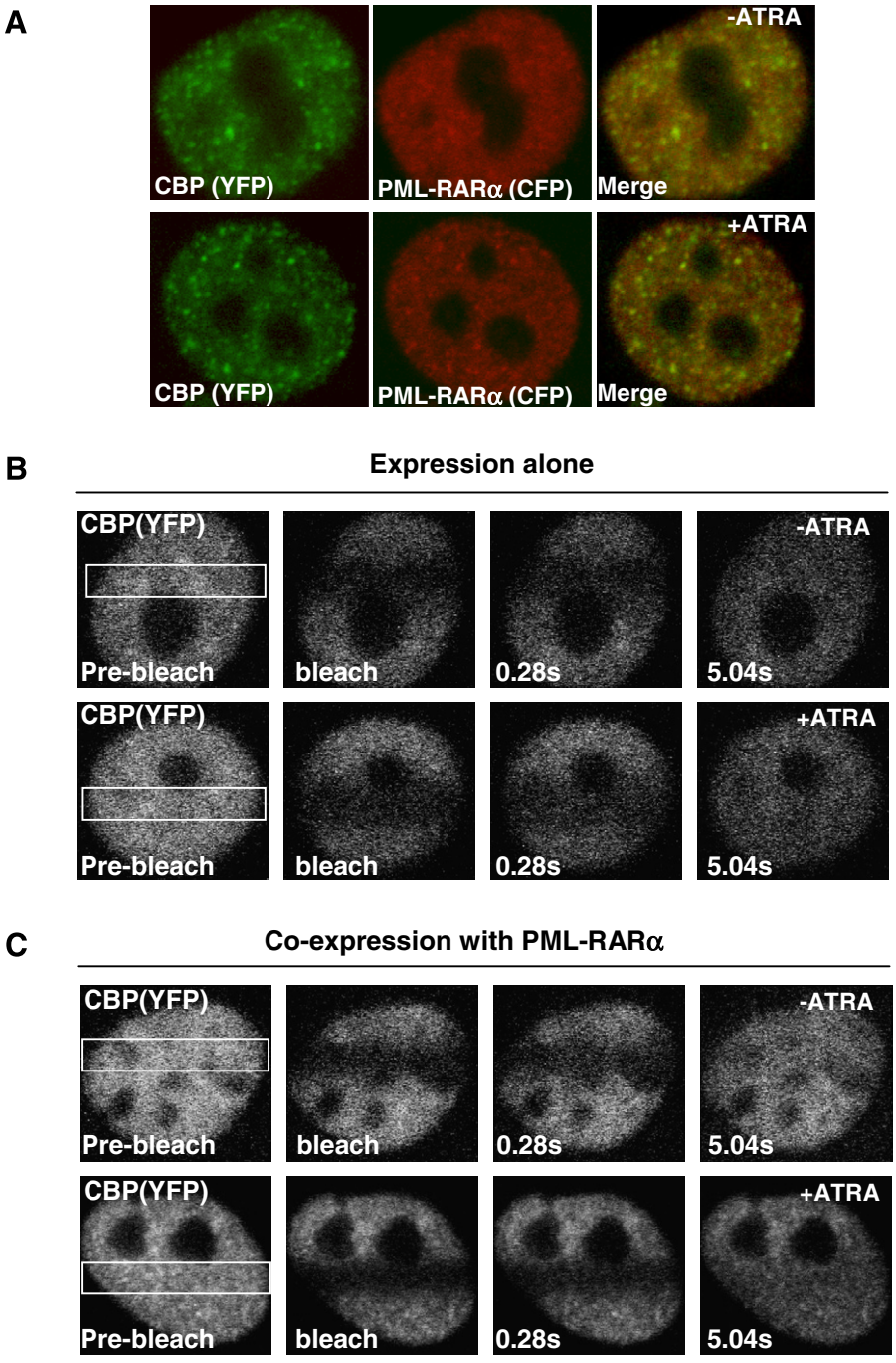

Fig. 4. FRAP analysis of YFP-CBP co-expressed with or without CFP-PML-RARα in the absence and presence of ATRA. (A) Co-localization of YFP-CBP and CFP-PML-RARα within the nucleus assessed by fluorescence microscopy in the absence and presence of ATRA. HeLa cells were co-expressed with YFP-CBP and CFP-PML-RARα and incubated with or without 10^{-6} M ATRA. FRAP analysis of YFP-CBP was performed on HeLa cells transfected with YFP-CBP alone (B) or co-transfected with YFP-CBP plus CFP-PML-RARα (C). Representative images show a single z-section obtained before photobleaching (Pre-bleach), at the end of photobleaching (bleach), and at the indicated time points after photobleaching. The white rectangle indicates the area bleached within the HeLa cells.

mobility of the transcriptional coactivator cAMP response element-binding protein (CBP), which possesses a histone acetyltransferase activity, was first demonstrated as a coactivator for general transcription machinery and subsequently has been shown to interact with numerous transcription factors, including nuclear receptors, to regulate the transcriptional activity. Therefore, we began to explore the effect of PML-RARα on CBP dynamics by FRAP analysis (Fig. 4 and Table 2). As shown in Fig. 4, YFP-CBP expression alone distributed preferentially within nucleus in a diffusion pattern and had a very fast recovery $t_{1/2}$ of 1.02 ± 0.35 and 1.06 ± 0.32 s in the absence and presence of ATRA, respectively ($P = 0.38$). However, unlike transcriptional co-repressor SMRT, YFP-CBP localized into the microspeckled structure occupied by CFP-PML-RARα examined by confocal microscopy in a hormone-independent manner, consistent to a recently published report [16]. Furthermore, we found that this co-localization of YFP-CBP and CFP-PML-RARα did not significantly alter the intranuclear mobility of YFP-CBP, with a recovery $t_{1/2}$ of 0.88 ± 0.28 and 0.90 ± 0.26 s in the absence and presence of ATRA ($P = 0.44$), respectively, suggesting that the constitutive interaction of CBP and PML-RARα had no effects on the intranuclear mobility of CBP.

Discussion

The mis-localization and reduced intranuclear mobility of PML-RARα oncogenic protein have been suggested to have a contribution on the pathogenesis of APL [10]. In present studies, we have found that the oligomerization-motif, coiled-coil domain of PML, not the single sumoylation site, is responsible for the abnormal nuclear localization and intranuclear mobility of PML-RARα.

Transcription factors must bind DNA and interact with other regulatory proteins, including the RNA polymerase basal machinery, co-activators or co-repressors and chromatin-remodeling complexes, in order to localize to their target gene within nucleus to regulate transcription. These functions are potentially subject to regulation by SUMO modification [5]. PML-RARα retains the sumoylation site within PML moiety and can be efficiently sumoylated by SUMO-1 as PML [8,17]. Functionally, the specific sumoylation site on lysine 160 residue in the PML moiety of PML-RARα was critical for APL phenotype such as differentiation arrest and immortalization [7]. One group has reported that desumoylation of PML-RARα led to the relocalization of PML-RARα into a nuclear diffuse form from the microspeckled structure [14], strongly implying the relationship between sumoylation and PML-RARα microspeckle formation. However, in our study, we provide the direct evidence at a single live cell level, that each known sumoylation site of PML-RARα is not responsible for its abnormal microspeckled distribution and reduced intranuclear mobility. Considering that these single-site mutants could not efficiently inhibit the total production of sumoylated PML-RARα as shown in a previous report [13], further confirmation of whether sumoylation of PML-RARα contributes to the aberrant dynamics of this fusion protein will be processed in the future. Intriguingly, the coiled-coil domain within PML moiety of PML-RARα, which was reported to be essential for PML-RARα oncogenic potential in APL involved homo-oligomerization, specific microspeckled localization and inhibition of monocyte differentiation [14], was responsible for mislocalization and intranuclear immobilization of oncogenic PML-RARα protein.

The interaction of PML-RARα and transcriptional co-repressor SMRT has been suggested to play the leading role in the initiation and development of APL [1–3]. In our present study, we further provided a direct and temporal dynamics of the interaction of PML-RARα and SMRT at a single cell level. In the absence of ATRA, SMRT and PML-RARα were shown to dynamically co-localize within the subnuclear foci and this co-localization led to a dramatically reduced mobility of SMRT in an immobilization fashion mirrored with that of PML-RARα. ATRA addition led to the dissociation of SMRT from PML-RARα foci and relieved the sequestration of SMRT from PML-RARα with the fluorescence recovery. While, RARα was shown as a mobile molecule reported by us previously [10], PML-RARα could efficiently immobilized SMRT in the microspeckled foci. The sequestration of co-repressor SMRT in the PML-RARα-occupied address will dramatically reduce the availability of SMRT to interact with other important transcriptional factors and subsequently disrupt their normal function, further highlighting a critical role for the reduced intranuclear mobility of PML-RARα in the pathogenesis of APL.

It has been reported that the agonist (oestradiol, E2) leads to the recruitment of nuclear receptor co-activator SRC-1 to the estrogen receptor (ER) nuclear foci and further reduced the intranuclear mobility of SRC-1 paralleled with the slower mobility of ER [12]. Fluorescent microscopic studies of cells expressing GFP-tagged CBP have shown that CBP was co-localized with androgen receptor (AR) in a ligand-dependent manner [18]. However, in our studies, the significant co-localization between YFP-CBP and CFP-PML-RARα within nucleus was identified regardless of absence or presence of ATRA following co-transfection with these two different fluorescent protein labeled constructs, suggesting that CBP could interact with PML-RARα in a ligand-independent way. Unexpectedly, unlike co-repressor SMRT, this localization of CBP into the address of PML-RARα has no effect on the intranuclear mobility of CBP. In line with our study here, we and others have reported that PML-RARα interacts with CBP and SRC-1 in a hormone-independent manner, thus attenuating the transcription of some target genes [11,16]. Taken together, these findings further imply the hormone-independent association between PML-RARα and co-activators might contribute to the aberrant regulation of the proper gene expression, which may be linked to the leukemogenesis.

Acknowledgments

We thank Drs. Qian Zhao and Lishun Wang for useful discussion. This work was supported in part by National Natural Science Foundation of China (30470360) (to S.D.).

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