Contribution of the C-terminal Regions of Promyelocytic Leukemia Protein (PML) Isoforms II and V to PML Nuclear Body Formation*

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Promyelocytic leukemia protein (PML) nuclear bodies are dynamic and heterogeneous nuclear protein complexes implicated in various important functions, most notably tumor suppression. PML is the structural component of PML nuclear bodies and has several nuclear splice isoforms that share a common N-terminal region but differ in their C termini. Previous studies have suggested that the coiled-coil motif within the N-terminal region is sufficient for PML nuclear body formation by mediating homo/multi-dimerization of PML molecules. However, it has not been investigated whether any of the C-terminal variants of PML may contribute to PML body assembly. Here we report that the unique C-terminal domains of PML-II and PML-V can target to PML-NBs independent of their N-terminal region. Strikingly, both domains can form nuclear bodies in the absence of endogenous PML. The C-terminal domain of PML-II interacts transiently with unknown binding sites at PML nuclear bodies, whereas the C-terminal domain of PML-V exhibits hyperstable binding to PML bodies via homo-dimerization. This strong interaction is mediated by a putative α-helix in the C terminus of PML-V. Moreover, nuclear bodies assembled from the C-terminal domain of PML-V also recruit additional PML body components, including Daxx and Sp100. These observations establish the C-terminal domain of PML-V as an additional important contributor to the assembly mechanism(s) of PML bodies.

PML3 nuclear bodies are dynamic subnuclear spherical protein domains with a typical diameter of 0.2–0.5 μm (1). These protein domains are highly regulated and are involved in a wide range of important cellular functions, including tumor suppression, transcriptional regulation, apoptosis, senescence, the DNA damage response, and viral defense mechanisms (2–7). Disruption of PML nuclear bodies is closely related to tumorigenesis. One such example is acute promyelocytic leukemia in which the formation of PML-RARα fusion proteins resulting from the reciprocal t(15;17) chromosomal translocation leads to disruption of normal PML-NBs and tumor formation (8, 9). In addition, PML nuclear bodies are often degraded or disintegrated upon viral infection, suggesting a role in cellular viral defense pathways (10–13). Thus, the integrity of PML nuclear bodies is of great importance in execution of their functions.

PML nuclear bodies are assembled through various interactions among PML protein isoforms. PML is the essential structural component of PML nuclear bodies (14). It belongs to the so-called TRIM protein family, which is characterized by a tripartite motif called TRIM or RBCC that includes a Ring finger, one or two B-boxes, and a Coiled-Coil motif (15, 16). The coiled-coil motif mediates a strong homo/multidimerization activity essential for core assembly of PML-NBs (15, 17, 18). Non-covalent interactions between sumo-conjugated PML and its sumo-interacting motif (SIM) allows the initial PML network to “mature” into larger scaffolds. The co-existence of sumoylation sites and SIM in PML is crucial not only for the formation of the mature and functional PML-NBs but also for providing binding sites for nuclear proteins with a similar architecture, most prominently Sp100 (14, 19–21). A few constitutive and more than 100 transiently binding proteins have been found to be localized to PML-NBs, many of which are mediated by the SUMO-SIM interaction network (22, 23). As shown for most subnuclear domains (24), PML bodies assemble through self-organizing and self-propagating mechanisms (25).

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2 The abbreviations used are: PML, promyelocytic leukemia protein; SIM, sumo-interacting motif; NLS, nuclear localization sequence; NB, nuclear body; MEF, mouse embryonic fibroblast; TRITC, tetramethylrhodamine isothiocyanate; FRAP, fluorescence recovery after photobleaching; RICS, raster image correlation spectroscopy.
The PML protein can be expressed as at least seven alternatively spliced isoforms. Except for isoform VII, which is cytoplasmic, PML isoforms I–VI are nuclear. These six nuclear PML isoforms share the same N-terminal region spanning amino acids 1–570 encoded by exons 1–6 and part of exon 7, which contains the RBCC motif and a nuclear localization sequence (NLS) (15, 26). However, they differ in their C termini due to alternative splicing from exons 7 to 9 (Fig. 1A). PML-VI is the shortest nuclear isoform encoded only by exons 1–6; hence, it does not have a distinctive C terminus (Fig. 1A). All PML nuclear isoforms co-exist at PML nuclear bodies in cultured as well as primary cells (27).

Increasing evidence suggests that the individual PML isoforms possess distinctive functions. PML-I and -II are considered to be the most abundant isoforms, and both are involved in restriction of virus replication (28–31). The C terminus of PML-II provides a binding site for adenovirus type 5 E4 Orf3 protein. This interaction mediates the virus induced PML-NB reorganization and disruption (32, 33). PML-III plays a disputed role in centrosome duplication and genome stability (34, 35). PML-IV is the mostly studied PML isoform. It participates in the regulation of cellular senescence, the DNA damage response, telomerase activity, and viral protein sequestration by specifically interacting with many important proteins such as p53, Mdm2, Tip60, and TERT (telomerase reverse transcriptase), suggesting the importance of its C terminus in mediating specific protein interactions (4, 36–39). PML-V shows a 5-fold slower exchange rate at PML bodies than other isoforms, suggesting that its C terminus may contribute to PML body stability (40).

The individual PML isoforms may also regulate the localization and morphology of PML-NBs (27, 41, 42). Expression of individual PML isoforms in various cellular backgrounds exhibited distinct nuclear localization patterns and can considerably alter the structure of PML bodies (24, 25). For example, the appearance and function of PML nuclear bodies at very early stages of mouse embryo development may depend on the expression of specific PML isoforms (43). Molecular analysis revealed that the C-terminal region of PML-I contains a nucleolar targeting sequence and a nuclear export sequence. These motifs mediate the relocalization of PML nuclear bodies into nucleoli or the cytoplasm, respectively (43, 44). PML-IV and -V also contain a nucleolar targeting-like sequence (44). PML-II contains a second NLS at its C terminus (41). These data suggest that the C-terminal domains of PML isoforms may contribute to the functional and structural diversity of PML-NBs. However, the detailed mechanism is still lacking.

In this study we report that the C-terminal domain of PML-II and PML-V can target to PML-NBs independent of their N-terminal regions and that they form nuclear bodies in the absence of endogenous PML. The C-terminal region of PML-V contains a putative α-helix segment that exhibits a strong homodimerization activity. Moreover, the nuclear bodies formed by the C-terminal domain of PML-V can recruit multiple PML-NB-associated proteins. These results implicate PML-V as an important PML isoform regulating the composition, formation, and stability of PML nuclear bodies.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Medium**—H1299, HEK293T cells, and PML−/− mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator supplied with 5% CO2.

**Chemicals and Antibodies**—Dabco 4’,6-diamidino-2-phenylindole (DAPI) was purchased from Beyotime. Antibodies were used against p53, Mdm2, Tip60, and TERT (telomerase reverse transcriptase), indicating the importance of its C terminus in mediating specific protein interactions (4, 36–39). PML-V shows a 5-fold slower exchange rate at PML bodies than other isoforms, suggesting that its C terminus may contribute to PML body stability (40).

**Yeast-directed Binding Assay**—The yeast two-hybrid approach we used to analyze protein interaction was described in (48). Briefly, the pACT2-PML-CT2 or -CT5 plasmid was co-transformed into AH109 with the pGBK7T7 plasmids expressing the C-terminal fragments of PML isoforms and PML-VI according to standard procedures. Empty vectors were served as negative controls. The presence of “bait” and “prey” proteins in co-transformed cells was indicated by growth on double drop-out plates devoid of leucine and tryptophan in the media. The interactions were scored positive if the co-transformed cells were also able to grow on quadruple drop-out plates devoid of leucine, tryptophan, histidine, and adenine.

**FRAP Assay**—Fluorescence recovery after photobleaching (FRAP) experiments were performed on a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) essentially as described before (40). 2–10 images were taken before the bleach pulse, and 50–200 images were acquired after bleaching of regions of interest containing one nuclear body each at 0.05%
laser transmission to minimize scan bleaching. Image acquisition frequency was adapted to the recovery rate of the respective GFP fusion protein. The pinhole was adjusted to 1 airy unit. Quantitation of relative fluorescence intensities was done according to Weidtkamp-Peters et al. (40) using Origin software (OriginLab, Northampton, MA).

Raster Image Correlation Spectroscopy (RICS)—The principles of RICS have been described in detail previously (49). In RICS, intensity fluctuations between neighboring pixels from confocal images are analyzed by spatially auto-correlating the image in the x and y direction using two-dimensional fast Fourier transformation (49). For RICS of the GFP fusion constructs in this study, time series of GFP fluorescence images were acquired in a subregion of the nucleus by confocal microscopy using a LSM710, the Plan-Apochromat 63× oil objective, and the RICS software package from Zeiss. Subregions (64×64 pixels) within this time series are then extracted, and correlation spectra are assessed from these subregions. Diffusion coefficient maps were generated by fitting with a free diffusion model assuming one component according to the RICS protocols provided by the manufacturer.

Western Blotting and in Vitro Immunoprecipitation—The cell lysates were made in lysis buffer (50 mM Tris-Cl at pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 10% glycerol, 20 mM NaF, 1 mM DTT, and 1× complete protease mixture). Protein samples were resolved by SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked in 5% skim milk and probed with the indicated antibodies. Detection was performed using an ECL detection kit (Cwbiotech). To analyze the interaction between the C-terminal domain of PML-II and that of individual PML isoforms as well as PML-VI, F-PML-CT2, individual GFP-nls-CT (1–5), and GFP-PML-VI were expressed separately in HEK293T cells. The cell extracts were prepared using lysis buffer as described previously. The amounts of GFP-tagged proteins in the soluble extracts were adjusted to the similar level by varying the amounts of the plasmids transfected. Fixed volumes of F-PML-CT2 and the control extracts were mixed with the individual GFP-tagged protein containing extracts. The immunoprecipitation was then carried out with anti-FLAG M2 beads for 30 min. The immunoprecipitated complexes were resolved by SDS-PAGE and immunoblotted with anti-GFP.
Cell Fractionation and Solubility Analysis—H1299 cells were transfected with plasmids expressing full-length and C-terminal region of PML-V as well as their respective Arg to Pro mutants. 24 h later the cells were lysed with the above 1% Triton X 100-containing lysis buffer for 20 min on ice. The cell lysates were then centrifuged at 13,800 g for 20 min. The supernatants were removed and designated as soluble fractions. The pellets were washed 3 times with PBS and then sonicated briefly on ice in the lysis buffer. The resulting samples were considered as insoluble fractions. Both soluble and insoluble fractions were subject to SDS-page and Western blot analysis.

RESULTS

The C-terminal Region of PML Isoforms II and V Bind to PML-NBs Independent of the Shared N-terminal Region—To assess the potential contribution of the C-terminal region of PML-V as well as their respective Arg to Pro mutants, H1299 cells were transfected with plasmids encoding the deletion mutants of GFP-CT2 for 24 h. The localizations of these mutants were detected by GFP fluorescence, and cell nuclei were stained with DAPI. Colocalization of GFP-CT2(651–690) with PML-NBs is shown. H1299 cells were transfected with the GFP-CT2(651–690) plasmid. After 24 h, PML-NBs were immunostained with a PML antibody. Endo. PML, endogenous PML. Scale bar, 10 μm.

FIGURE 2. Critical region of CT2 is mapped for PML-NBs targeting. A, shown is a schematic diagram of the deletion mutants of GFP-CT2. The mapped PML-NB targeting region was indicated by a gray rectangle. B, H1299 cells were transfected with the plasmids encoding the deletion mutants of GFP-CT2 for 24 h. The localizations of these mutants were detected by GFP fluorescence, and cell nuclei were stained with DAPI. C, colocalization of GFP-CT2(651–690) with PML-NBs is shown. H1299 cells were transfected with the GFP-CT2(651–690) plasmid. After 24 h, PML-NBs were immunostained with a PML antibody. Endo. PML, endogenous PML. Scale bar, 10 μm.

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RESULTS

The C-terminal Region of PML Isoforms II and V Bind to PML-NBs Independent of the Shared N-terminal Region—To assess the potential contribution of the C-terminal region of PML isoforms I-V to the localization of PML nuclear bodies, we fused them individually to GFP containing a NLS. These fusion proteins were designated as GFP-nls-CTn (n = 1–5) (Fig. 1B). We then examined the subcellular localization of these fusion proteins in H1299 cells. GFP-nls-CT3 and GFP-nls-CT4 localized exclusively in a diffuse pattern throughout the nucleoplasm excluding nucleoli. GFP-CT2 was still able to form nuclear dots probably due to the NLS at its C terminus (39). GFP-CT5 tended to appear as aggregated dots in the cytoplasm and GFP-CT1, CT3, and CT4 distributed diffusely throughout the cells. Similar results were also obtained in HEK293T and HEp-2 cells (data not shown).

A previous report had indicated that the C-terminal region of PML-I and -V may contain a nucleolar targeting sequence (44). We, therefore, performed immunostaining to determine whether the nuclear speckles formed by CT1, CT2, and CT5 represent nucleoli. Not surprisingly, GFP-nls-CT1 nuclear speckles were completely colocalized with UBF, a nucleolar fibrillar center marker, whereas those of GFP-CT2 were not (Fig. 1D). Few GFP-nls-CT5 speckles were found in the nucleolar area but not co-localized with UBF, suggesting that these speckles do not localize in the fibrillar center within nucleoli (Fig. 1D, g–i). Because the size and number of the nuclear dots formed by GFP-CT2 and GFP-nls-CT5 were similar to those of PML-NBs, we tested this possibility by co-immunostaining of endogenous PML bodies. Strikingly, while GFP-nls-CT1 does not overlap with PML-NBs, both GFP-CT2 and GFP-nls-CT5 colocalized strongly with PML-NBs (Fig. 1E). These data clearly show that the C-terminal regions of PML-II and V have the ability to target to PML-NBs independent of the N-terminal RBCC region.
**PML Body Targeting Regions within CT2 and CT5**—To map the region in PML CT2 responsible for targeting to PML-NBs, we made a series of deletion mutants and then examined their subcellular localization (Fig. 2A). Through this approach, we determined the PML-NB targeting region of PML CT2 to a 40-amino acid segment ranging from residue 651 to 690 of the PML-II protein sequence (Fig. 2B). When fused to GFP, this region induced formation of nuclear dots, which fully colocalized with endogenous PML-NBs (Fig. 2C). In contrast, PML-CT2 deletion variants devoid of this 40-amino acid region completely lost the ability to form nuclear dots even when fused to a NLS (Fig. 2B, f and g).

Using a similar approach, we found that a 21-amino acid-long region within PML-V (591–611) is sufficient for nuclear dot formation of GFP-nls-CT5 (Fig. 3A). The gray rectangle indicates the critical PML-NB targeting region of PML-V\textsubscript{591RLAL603} (B). H1299 cells were transfected with the expression plasmids for GFP-nls-CT5 wild type or mutant proteins. 24 h later images were taken by fluorescence microscopy. Cell nuclei were stained with DAPI. Scale bar, 10 μm. C and D, H1299 cells were transfected with GFP-nls-CT5 and GFP-nls-CT5(R/P) plasmids (C) or full-length FLAG-PML-V and FLAG-PML-V(R/P) plasmids (D). 24 h later the cells were lysed and fractionated into the soluble and insoluble fractions. The distribution of the expressed proteins were analyzed by Western blot with the antibodies against GFP (C), and FLAG (D), α-tubulin (soluble fraction marker (S)), and HP-1 (insoluble fraction marker (IN)). R/P refers to GFP-nls-CT5 or FLAG-PML-V mutant in which Arg residues 599 and 603 of PML-V were mutated to prolines.

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**PML-CT2 Is Preferentially Recruited to Nuclear Bodies by PML Isoform II**—To determine whether the localization of the C-terminal domain of PML-V is mediated by interaction with any of the six nuclear PML isoforms, we examined the nuclear localization of GFP-CT2 dependent on co-expression of full-length PML isoforms (Fig. 4A). In PML-proficient H1299 cells, the C-terminal domain of PML-II was efficiently recruited to nuclear bodies containing overexpressed PML-II, -IV, -V, and -VI (Fig. 4A, B and D–F). GFP-CT2 also formed individual nuclear assemblies in PML-I and PML-III-overexp-
A. H1299 were co-transfected with the expression plasmids for GFP-CT2 (CT2, green) and individual RFP-PML isoforms (PML-I to PML-VI, red). At 24 h after transfection, GFP, RFP, and DAPI signals were acquired by fluorescence microscopy. The left panel shows merged images of the red and green channels, and the right panels show the DAPI signal in monochrome. Colocalization in nuclear bodies (Coloc. in NBs) was defined as full overlap of green and red dot-like structures in PML bodies. Insets in b and d–f show magnified monochrome views of the green and the red channels from areas containing a PML body (depicted by the white box). Scale bar, 10 μm. B. PML−/− MEFs were co-transfected with the expression plasmids for GFP-CT2 and individual FLAG-tagged PML isoforms (PML-I to PML-VI). At 24 h after transfection, FLAG-tagged PML isoforms were immunostained with an anti-PML antibody (red). The cells were then visualized and analyzed as described for A. The insets in b show magnified monochrome views of the green and the red channels from areas containing a PML body (depicted by the white box). Scale bar, 10 μm. C. FLAG-PML-CT2 and equal amounts of GFP, individual GFP-nls-CT (1–5), and GFP-PML-VI proteins were expressed in 293T cells. The cell extracts containing GFP or individual GFP fusion proteins were mixed with the fixed volumes of the cell extracts transfected with FLAG-PML-CT2 expression plasmid or the control empty FLAG vector plasmid. FLAG-PML-CT2-binding complex was immunoprecipitated (IP) with anti-FLAG M2 beads and analyzed by Western blot with anti-GFP and anti-FLAG antibodies.
pressing cells, but these structures, although frequently associated with the PML nuclear bodies, did not fully colocalize with them (Fig. 4A, a and c). The same results were obtained in the PML-proficient cell line HEp-2 (data not shown). In PML null MEFs, however, the C-terminal region of PML-II was only efficiently recruited into NBs containing PML-II (Fig. 4Bb), whereas there was no colocalization between CT2 dots and nuclear bodies consisting of the other full-length PML isoforms (Fig. 4B, a and c–f). These observations strongly suggest that the C-terminal domain of PML-II can specifically self-interact at nuclear bodies even in the absence of other PML isoforms. Consistent with this conclusion, the presence of endogenous PML-II in H1299 cells may facilitate recruitment of PML-CT2 to PML-NBs, with the notable exception of nuclear bodies containing overexpressed full-length PML-I or PML-III (Fig. 4A, a and c). One possible explanation for this observation may be that overexpression of PML-I or PML-III or other cellular factors specifically recruited by these isoforms somehow interfere with the CT2 self-interaction activity. To further examine the mechanism that governs the targeting of PML-CT2 to PML-NBs, we employed a yeast two-hybrid approach and immunoprecipitation assays. PML-CT2 failed to interact with any of the C-terminal regions of PML isoforms and PML-VI in a yeast two-hybrid analysis (data not shown) but could specifically pull down itself, PML-CT4, PML-CT5, and full-length PML VI (Fig. 4C). These immunoprecipitation results are fully consistent with the colocalization data in PML-proficient cells (Fig. 4A).

The C-terminal Region of PML-V Interacts with PML Isoforms IV and V—Similarly, we examined the relationship between PML-CT5 and the different PML isoforms. In PML-proficient H1299 cells, GFP-nls-CT5 was efficiently recruited to nuclear bodies containing overexpressed PML-III, -IV, and -V (Fig. 5A, c–e) but not to nuclear bodies containing the other PML isoforms in excess amounts (Fig. 5A, a, b, and f). In PML null MEFs, nls-CT5 was exclusively recruited to nuclear bodies consisting of PML-IV or PML-V (Fig. 5B, d and e, respectively). These observations suggest that the C-terminal domain of PML-V can directly interact with the C-terminal domains of PML-IV and -V. In the PML-proficient cell line HEp-2, nls-CT5 was also exclusively recruited into PML-IV and PML-V overexpressing PML bodies (data not shown). Therefore, the accumulation of nls-CT5 at PML bodies of H1299 cells overexpressing PML-III (Fig. 5Ac) may be explained by an additional cellular factor(s) that is not present in HEp-2 cells or in PML-null MEFs. Because endogenous PML-IV and -V should be present at PML nuclear bodies in PML-I, -II, or -VI overexpressing H1299 cells, one would expect that these endogenous molecules provide sufficient binding sites for nls-CT5 to accumulate at PML bodies. However, this was not observed (Fig. 5A, a, b, and f). Probably, overexpression of these full-length isoforms interfere with the direct interaction between nls-CT5 and endogenous PML-IV or -V.

As the high insolubility of PML-V in vitro made analyses of direct protein-protein interactions impossible (data not shown), we employed a yeast two-hybrid approach to further examine complex formation. This assay revealed binding of PML-CT5 to CT3, CT4, CT5, and full-length PML-VI (Fig. 5C). As a positive control for this assay, SUMO-1 was also found to form a complex with PML-VI in yeast cells (Fig. 5C). Taken together, the C-terminal region of PML-V is able to (i) homodimerize with itself, (ii) heterodimerize with the C terminus of PML-IV, and (iii) form a complex with PML isoforms III and VI.

The C-terminal Domain of PML-II Interacts Dynamically at PML Nuclear Bodies—Next, we used FRAP to further analyze the binding dynamics of the C-terminal domain of PML-II at PML-NBs in living cells. In FRAP, the fluorescence of GFP fusion proteins is bleached by a very short laser pulse, and fluorescence recovery into the bleached spot is then monitored over time. The shape of the resulting fluorescence recovery curve contains information on the diffusion and interaction dynamics of GFP fusion proteins. Fig. 6A shows a FRAP experiment of GFP-nls-CT2 at PML nuclear bodies co-expressing full-length RFP-tagged PML isoform II. Fluorescence of GFP-nls-CT2 at nuclear bodies in the absence of other isoforms recovered rapidly within seconds, indicating a high exchange rate of this fusion protein at endogenous PML bodies (data not shown). Similar FRAP experiments were also performed in the presence of overexpressed full-length RFP-tagged PML isoforms I-VI, and the resulting FRAP curves are shown in Fig. 6B. These data revealed a highly dynamic exchange of GFP-nls-CT2 at PML nuclear bodies independent of the overexpressed full-length PML isoform. Recovery curves returned to prebleach values within 10–20 s, indicating that the complete pool of GFP-nls-CT2 has turned over at PML bodies during that time (Fig. 6B). Thus, the C terminus of PML-II can transiently interact with as yet unknown binding sites at PML nuclear bodies.

The C-terminal Domain of PML-V Can Form Hyperstable Scaffolds at PML Bodies—Binding of the C-terminal domain of PML-V to PML bodies was also analyzed by FRAP. Fluorescence recovery of GFP-nls-CT5 at PML bodies was slow in the absence and in the presence of co-expressed RFP-PML-V (Fig. 7A and data not shown). The mutant construct GFP-nls-CT5(R/P) localized only diffusely in the nucleoplasm of living cells (Fig. 7B), consistent with our previous observation (Fig. 3B). Even the presence of overexpressed RFP-PML-V did not recruit visible amounts of GFP-nls-CT5(R/P) into PML bodies (Fig. 7B). FRAP of this construct was rapid and complete within several seconds, indicating free diffusion of the mutant in the nucleus (Fig. 7, B and C, brown graph). Thus, arginine at positions 599 and 603 of PML-V is absolutely required for the self-interaction activity of the C-terminal domain of PML-V at nuclear bodies in living cells.

Quantification of the FRAP experiments revealed a very slow exchange of GFP-nls-CT5 at PML bodies in the presence of RFP-PML-V (Fig. 7C, red curve). Previously we had observed that GFP-PML-V has a very long residence time (48 min) at PML bodies (39). Consistent with this we observed here by FRAP a steady but slow recovery of RFP-PML-V (Fig. 7C, black curve). When GFP-nls-CT5 was co-expressed, fluorescence recovery of full-length RFP-PML-V was significantly reduced (Fig. 7C, blue curve). Moreover, the FRAP curves of RFP-PML-V and GFP-nls-CT5 were almost identical in coexpressing cells (Fig. 7C, compare red and blue curves). These results indicate that the C-terminal domain of PML-V has a strong homo-oligomerization activity in living cells that is able to induce hyperstable retention at nuclear bodies. To confirm the
specificity of the homo-oligomerization activity of CT5, we directly compared by FRAP the dynamic exchange of GFP-nls-CT5 in the presence of overexpressed PML-IV and PML-V. This experiment confirmed immobilization at PML bodies of GFP-nls-CT5 in the presence of PML-V, whereas this construct exhibited more dynamic exchange at PML bodies in the presence of overexpressed PML-IV (Fig. 7, D–F). FRAP analyses of GFP-CT5 at PML bodies containing overexpressed RFP-tagged PML-I, -II, -III, and -VI showed similar recovery curves as the one observed for RFP-tagged PML-IV (data not shown).

To further characterize the scaffolding function of CT5, we fused this region to the C-terminal ends of full-length GFP-PML-I and GFP-PML-II and analyzed their dynamics by FRAP (Fig. 8). Consistent with our previous observations (39, 50), GFP-tagged PML-I and GFP-PML-II dynamically exchanged at nuclear bodies over a 10–20-min time period (Fig. 8C). FRAP
recovery was significantly reduced by fusing the C-terminal domain of PML-V to the C terminus of these proteins (Fig. 8C). This observation shows that this domain exerts an immobilizing property that competes with and overrides the binding properties of wild-type PML-I or PML-II at nuclear bodies. The immobilizing property of CT5 became even more evident when RFP-PML-V was co-transfected. In this setting GFP-tagged PML-I-CT5 and PML-II-CT5 showed hyperstable retention at nuclear bodies (Fig. 8C). Taken together the FRAP results show that the C-terminal domain of PML-V can function as a PML body retention signal through its strong homo-oligomerization activity.

**The C-terminal Domains of PML Isoforms I-V Have Binding Sites Outside Nuclear Bodies**—Although suggested by analyses of PML isoform-specific functions, there is no experimental proof if PML isoforms bind to nucleoplasmic interaction partners via their C-terminal domains outside of nuclear bodies. To address this question, we used RICS. RICS allows determination of the diffusion coefficient of GFP fusion proteins in different subregions of living cells (51). Fig. 9, A–C, shows a typical RICS analysis of GFP-nls-CT2 in a subregion of the nucleus. From this analysis, a diffusion map was generated that displays the diffusion coefficient of GFP-nls-CT2 in the region of the nucleus selected for RICS imaging (Fig. 9D). In the same way, RICS was performed for all GFP-tagged C-terminal domains and for GFP alone in the nucleus excluding nuclear bodies (Fig. 9E). We determined by RICS in HEp-2 cells a diffusion coefficient for GFP alone of $D = 23 \pm 6 \mu m^2 s^{-1}$ in the nucleus, consistent with previously published data using related fluorescence fluctuation techniques (52). The diffusion coefficients of the GFP-tagged C-terminal domains of PML isoforms I-V as measured by RICS were considerably lower than $D$ of GFP alone (Fig. 9E). The reduced mobility of the GFP-CTs was not caused by their increased molecular weight compared with GFP.

**FIGURE 6.** The C-terminal domain of PML-II exchanges rapidly at PML bodies independent of cotransfected full-length PML isoforms. A, FRAP was performed in circular regions containing a PML body in living HEp-2 cells co-expressing GFP-nls-CT2 and RFP-PML-II. Images show GFP and RFP fluorescence before (pre) and after (post) the 488-nm laser bleach pulse and at different time points later. Bar, 10 \( \mu \)m. B, FRAP of GFP-nls-CT2 fluorescence was quantitated from experiments as shown in A in the presence of overexpressed RFP-tagged full-length PML isoforms as indicated. Graphs show mean values (\( n \geq 10 \)). S.D. were less than 10\%. RFI, relative fluorescence intensity.

**FIGURE 7.** The C-terminal domain of PML V stably binds to PML bodies. A, shown is a FRAP experiment of GFP-nls-CT5 (green) at PML bodies in HEp-2 cells coexpressing RFP-PML-V (red). B, shown is FRAP experiment of GFP-nls-CT5 (R/P) (green) at PML bodies in HEp-2 cells coexpressing RFP-PML-V (red). C, shown is quantitation of FRAP experiments of different fusion proteins as indicated in the legend below the graphs. D, FRAP of GFP-nls-CT5 in cells coexpressing RFP-PML-IV is shown. E, FRAP of GFP-nls-CT5 in cells coexpressing RFP-PML-V. F, shown is quantitation of FRAP of GFP-nls-CT5 fluorescence in the presence of overexpressed RFP-PML-IV (black curve) or RFP-PML-V (red curve). Graphs show medium values from FRAP experiments performed in at least 10 cells; S.D. is also shown in C. S.D. in F were less than 12\%. RFI, relative fluorescence intensity. Bars, 10 \( \mu \)m.
alone (Fig. 9E, hatched bars). Therefore, the decreased diffusion coefficients of GFP-CT 1–5 can only be the result of incorporation into larger and, therefore, slower moving complexes or transient interactions with immobile chromatin or both. Interestingly, even the mutated GFP-CT5 construct, which does not bind to PML bodies, has a substantially decreased diffusion coefficient (i.e., has binding sites) throughout the nucleoplasm (Fig. 9E). These observations provide the first experimental evidence that the C-terminal domains of the nuclear PML isoforms I–V have binding partners in the nucleus outside nuclear bodies.

**The C-terminal Region of PML-V Is Sufficient to Recruit Multiple PML Body Components**—The FRAP results suggested that CT5 may have a strong nucleating ability. Indeed, GFP-nls-CT5 can form NBs in PML null MEFs (Fig. 10A). To assess whether the C-terminal region of PML-V may play a role in the assembly of mature PML-NB protein complexes, we cotransfected GFP-nls-CT5 along with Daxx or Sp100, two well established PML-NB-associated proteins, in PML null MEFs. Consistent with previous observations (14, 20), overexpressed Daxx and Sp100 was found almost exclusively in a diffuse distribution pattern throughout the nucleus of most PML-negative cells, although in some cells these proteins were able to form a few dot-like structures (Fig. 10, B and C, a–c, respectively). However, co-expression of GFP-nls-CT5 induced the formation of many dot-like structures to which Daxx and Sp100 were efficiently recruited (Fig. 10, B and C, d–f, respectively). These observations indicate that the C-terminal region of PML-V can recruit typical PML body components independently of the N-terminal RBCC region. The C-terminal region of PML-II did not exhibit this activity (Fig. 10, B, g–i, and C, g–i).

The observed interaction between CT4 and CT5 (Fig. 5B) prompted us to examine the ability of the CT5-containing nuclear bodies to recruit PML-IV. To reduce the ability of PML-IV to form nuclear bodies through its RBCC region, we used PML-IV-m6, a mutant in which six cysteine residues within the RBCC region are mutated (Fig. 10D). As a result, PML-IV lost the ability to form typical PML-NBs (Fig. 10Da). However, when GFP-nls-CT5 was co-expressed in PML null MEFs, PML-IV-m6 assembled into nuclear bodies together with GFP-nls-CT5 (Fig. 10D, b–d). This effect of GFP-nls-CT5 is likely due to the interaction between CT4 and CT5, as it did not induce nuclear body formation in cells co-expressing PML-VI-m6 (Fig. 10D, e–h). These observations show that the C-terminal part of PML-V is able to specifically recruit full-length PML-IV via the unique C-terminal region of PML-IV and independent of its RBCC motif.

**DISCUSSION**

The current model suggests that PML body assembly and maintenance is driven by the RBCC motif in the N-terminal part of all PML protein isoforms and a SUMO-SIM interaction network (21, 26). In this study we discovered that the C-terminal regions of PML-II and PML-V can target to PML-NBs independent of the common N-terminal region. We provided strong evidence to suggest that the C terminus of PML-V contributes directly to the formation of PML nuclear bodies by mediating a homomeric protein oligomerization activity at nuclear bodies in living cells. The unique C terminus of PML-II also preferentially binds to full-length PML-II but can also transiently interact with the other isoforms at PML nuclear bodies. Our data extend the current model of PML NB formation and suggest that the C-terminal domains of PML-II and PML-V are two novel entities in regulating structural and functional properties of PML-NBs.

We showed in this study that the C terminus of PML-V can mediate homomeric interaction resulting in the formation in PML-negative cells of assemblies that resemble PML nuclear bodies with respect to size, shape, and biochemical composition. These observations strongly suggest that the C terminus of PML-V contributes to the assembly, maintenance, and structural stability of PML nuclear bodies. The responsible motif was identified to be the putative α-helical sequence of N-QQVTL-RLALRL-C. The α-helix destabilizing mutations Arg-599/603 to proline nearly abolished the ability of the C terminus of PML-V to form nuclear or cellular bodies, whereas exchange of Arg-599/603 to alanine had no visible effect (data not shown). Thus, it is conceivable that an α-helix motif exists in the C terminus of PML-V, which exhibits a strong homomeric interaction activity.
Strikingly, the self-interaction activity of the C terminus of PML-V results in extremely stable structures as we have shown here by FRAP. Our previous live cell imaging data had shown that PML-V has by far the longest residence time (48 min) of all PML isoforms at nuclear bodies in human cells (40). The data reported here not only confirm our idea that PML-V may serve a structural scaffold for PML bodies (24), but they also provide the molecular mechanism by which this scaffold function is realized. In PML-positive and PML-null MEFs, the residence time of PML-V at nuclear bodies is very similar, and again 2-3-fold stronger than the other PML isoforms (45). This observation suggests that the exchange rate of PML-V at nuclear bodies is mainly regulated by its unique C terminus. Among the six PML nuclear isoforms in human cells, only isoforms I and V are conserved between human and mouse (27). Thus, although expressed in low amounts (27), PML-V may substantially contribute to the structure and integrity as well as to the function of PML nuclear bodies. Low expression levels of PML-V (27) may even be required for normal nuclear body function as expression above a certain threshold may lead to insoluble aggregates, as suggested by our observations.

In addition to the homomeric interaction, we also observed that the C terminus of PML-V interacts with other PML isoforms. It binds to PML-VI and the C termini of PML-III and -IV. An interesting phenomenon is that the nuclear dots formed by the C terminus of PML-V are sometimes tightly associated but not completely colocalized with the structures formed by the other PML isoforms. Whether the spatial proximity of these two types of bodies is mediated by the interaction between the C terminus of PML-V and the N-terminal region of PML requires further investigation. Super-resolution microscopy has revealed that PML molecules are localized in patches of varying molecule concentration within a 50- to 100-nm-thick shell, which forms the surface of PML nuclear bodies (53). It is possible that PML-V overexpression leads to PML-V-specific aggregates within this shell. Such stable subregions at the surface of PML bodies may provide specialized scaffolds for specific biochemical reactions.

PML-IV is the most studied isoform. Its overexpression in human diploid fibroblast and PML<sup>+/−</sup> MEFs induces cellular senescence (4). Interestingly, PML-IV fails to trigger cellular senescence in PML<sup>−/−</sup> MEFs, suggesting that other PML isoforms may be required for this process. Our finding that PML-V can interact with PML-IV via its C terminus suggests that PML-V may assist the functions of PML-IV. PML-IV can recruit p53 to the PML nuclear bodies, where PML-p53-CBP complexes are assembled resulting in p53 acetylation and cellular senescence (54). It will be interesting to investigate whether the interaction between PML-V and PML-IV contributes to this process.

Recently it was shown that PML-V, but not the other PML isoforms, can bind to Rad51 and that homology-driven DNA repair becomes inefficient in the absence of PML bodies (55). Based on numerous reports, which functionally link PML bodies to DNA repair foci (56), it is tempting to speculate that PML-V functions at PML bodies to stabilize Rad51/DNA complexes at damaged chromatin. A stable interaction between PML-V and Rad51 at the surface of PML bodies could also explain the observation that single-stranded DNA is recruited to PML nuclear isoforms in human cells, only isoforms I and V are conserved between human and mouse (27). Thus, although expressed in low amounts (27), PML-V may substantially contribute to the structure and integrity as well as to the function of PML nuclear bodies. Low expression levels of PML-V (27) may even be required for normal nuclear body function as expression above a certain threshold may lead to insoluble aggregates, as suggested by our observations.

The mechanism responsible for the C terminus of PML-II localizing in PML nuclear bodies is probably different from that of PML-V. Although it can pull down itself in a co-immunoprecipitation assay and co-localizes only with PML-II but not with other PML isoforms in PML null MEFs, the C terminus of PML-II failed to interact with itself in a yeast two-hybrid analysis suggesting a lack of direct interaction. Therefore, a more likely mechanism is that some cellular components are involved in recruiting the C terminus of PML-II to PML nuclear bodies.
and even determines the localization of PML nuclear bodies. Depending on the cell type, PML-II accumulates either at the inner nuclear membrane or the central regions of the nucleus, indicating that the cellular components to which PML-II can specifically bind are differentially distributed in different cell types. Interestingly, in PML−/− MEFs, overexpressed PML-II can localize in both regions, but the C-terminal region of PML-II mainly targets to the central regions. Thus, the primary binding sites for the C-terminal region of PML-II are localized in the central region, and the N terminus of PML-II may contribute to the nuclear envelope localization of PML-II.

Increasing evidence suggests that the C termini of PML isoforms may play an important role in regulating the localization of PML nuclear bodies. When overexpressed in various cells, the different isoforms exhibited distinct localizations (27). However, when all isoforms co-exist in PML nuclear bodies, the

FIGURE 10. The C-terminal region of PML V forms NBs independent of endogenous PML that can recruit multiple PML-NB associated proteins. A, PML−/− MEFs were transfected with GFP-nls-CT (1–5) expression plasmids for 24 h. Images of transfected cells were taken by fluorescence microscopy. Cell nuclei were stained with DAPI. B and C, PML−/− MEFs were co-transfected with expression plasmids for GFP-nls, GFP-nls-CT5, and GFP-nls-CT2 alone or together with FLAG-Daxx (B) or RFP-Sp100 (C) plasmids. Images were taken by fluorescence microscopy. FLAG-Daxx was immunostained with an anti-FLAG antibody (B). RFP-Sp100 was detected by RFP fluorescence (C); co-localization was shown in yellow in the merge pictures. D, shown is a schematic diagram of PML-IV-m6 and PML-VI-m6 (top panel); Asterisks indicate point mutation. PML−/− MEFs were transfected with expression plasmids for FLAG-PML-IV-m6 and FLAG-PML-VI-m6 alone or together with GFP-nls-CT5. GFP-nls-CT5 was detected by the GFP fluorescence; FLAG-PML-IV-m6 and FLAG-PML-VI-m6 were immunostained with an anti-PML antibody. Colocalization is shown in yellow in the merge pictures. Scale bar, 10 μm.
dominant one may determine the localization. It has been previously shown that both PML-I and -IV contain a nucleolar targeting sequence (44); however, only PML-I, the major isoform, can direct the PML nuclear body to the nucleoli under stress conditions, which likely induce the exposure of the nucleolar targeting sequence of PML-I. PML-II is another major isoform (27), and the fact that the C terminus of PML-II can target to PML nuclear bodies highly suggests that PML-II can direct PML nuclear bodies to cellular sites to which its C terminus specifically binds.

The nuclear body-targeting region of PML-II lies between amino acids 651 and 690. This region is also attacked by the adenovirus 5-encoded protein E4 Orf3 during infection, resulting in relocalization and distortion of PML nuclear bodies (32, 33). Therefore, this region is an important functional domain of PML-II. Because both Orf3 and the cellular components bind the same region of PML-II, it would be interesting to determine whether the association of Orf3 with PML-II disrupts its interaction with the cellular components. Currently we do not know the identity of these cellular components, and using a yeast two-hybrid screen looking for the binding partners of the C-terminal region of PML-II amino acids 651–690 revealed that it contains a high number of positively charged amino acids, indicating its potential binding to transcriptional regulators. PML nuclear bodies are displayed electron-microscopically as hollow protein domains with threads sticking out making contacts with specific genomic loci (58, 59). We speculate that PML-II may be involved in connecting PML-NBs to specific genomic regions. Taken together our data provide strong evidence to suggest that the C termini of PML-II and PML-V are important new players in regulating the localization, assembly dynamics, and functions of PML nuclear bodies.

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