Analysis of the Growth and Transformation Suppressor Domains of Promyelocytic Leukemia Gene, PML*

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The promyelocytic leukemia gene (PML) involved in the t(15;17) (q22;q12) translocation in acute promyelocytic leukemia is a growth suppressor. To elucidate the functional domains of PML, several mutants lacking the nuclear localization signal (PMLnls−), the dimerization domain (PMLdim−), the proline-rich domain at the N-terminal (PMLpro−), the proline-rich RING finger motif (PMLpr−), the proline-rich RING finger B-box-1 (PMLprb−), the serine-proline-rich domain at the C-terminal (PMLsp−), and the double mutant (PMLprb−nls−) have been constructed. Immunofluorescence staining of transiently transfected NIH3T3 cells demonstrated that the RING finger motif, dimerization domain, and nuclear localization signal are all required for the formation of PML oncogenic domains (PODs). Immunofluorescence staining of transiently transfected GM637D human fibroblasts indicated that expression of PMLprb−, PMLnls−, and PMLprb−nls− led to a significant reduction or, in some cases, complete elimination of PODs. PMLdim−, PMLnls−, PMLpr−, PMLprb−, and PMLprb−nls− mutants were found to lose their ability to suppress transformation of NIH3T3 cells by activated neu, while PMLpro− and PMLsp− mutants did not. These results suggest that the ability of PML to form a POD is essential for suppression of growth and transformation. Furthermore, since PMLpr−, PMLnls−, and PMLprb−nls− mutants could block the suppression effect of wild-type PML on transformation of NIH3T3 cells by the neu oncogene, these PML mutants are potential dominant negative inhibitors of PML. Our study also suggests that the RING finger motif may interact with other nuclear proteins.

The nonrandom chromosomal translocation t(15;17) is a cytogenetic hallmark of acute promyelocytic leukemia (APL) (1), and the genes involved at the breakpoint site have been cloned and characterized (2–11). It is now clear that the t(15;17) translocation fuses the retinoic acid receptor-α (RARα) gene on chromosome 17 with the PML gene on chromosome 15. Fusion genes, PML-RARα and RARα-PML, transcribe fusion transcripts and encode potentially oncogenic fusion proteins. Since most of the functional domains of both PML and RARα are retained in the fusion protein PML-RARα, it is speculated that PML-RARα is critical for the pathogenesis of APL. Indeed, in cotransfection assays, it was found that PML-RARα represses RA-responsive promoters in the absence of RA (5–7, 9). The stable expression of PML-RARα inhibits apoptosis in U937 cells and makes these cells unable to respond to differentiation induction by 1,25-dihydroxyvitamin D3 (vitamin D3) and transforming growth factor β1 (12). In addition, stable expression of PML-RARα in K562 cells interferes with erythroid differentiation induced by hemin (13, 14). Thus, expression of this fusion protein in APL cells may be responsible for blocking differentiation and prolonging survival of promyelocytes.

PML-RARα can effectively sequester PML and the retinoic X receptor (RXR) (7, 15–17). In APL cells, double-color immunofluorescence staining of PML, RXR, and PML-RARα demonstrated that both PML and RXR colocalize with PML-RARα in vivo (16). However, after all-trans-retinoic acid (ATRA) induced differentiation of the APL cells, PML and RXR no longer colocalized with PML-RARα, indicating that sequestration of PML and RXR by the PML-RARα fusion protein is critical for the development of APL. This notion is strongly supported by our recent finding that PML is a growth suppressor (18). In brief, we found that (i) increased expression of PML in the APL cells results in a loss of donogenicity in soft agar assay and tumorigenicity in nude mice (18); (ii) PML suppresses the transformation of rat embryo fibroblasts by cooperative oncogenes and of NIH3T3 cells by activated neu (18, 19); (iii) PML is a promoter-specific transcription suppressor, suppressing the promoter activity of the epidermal growth factor receptor and multidrug-resistance genes and having little effect on the promoters of β-actin, SV-40, and Rous sarcoma virus (18). From this study and others, we hypothesized that PML-RARα plays a central role in APL as a dominant negative inhibitor of the normal function of PML (growth suppressor) and RXR (corepressor of RAR, thyroid hormone, vitamin D receptor, etc.) and consequently leads to the development of APL by growth stimulation and loss of differentiation induction by hormone or growth factors.

PML is a member of the family of RING finger proteins capable of forming a homodimer through the dimerization domain (20). As shown by immunofluorescence staining, PML accumulates in the nucleus in a speckled pattern composed of nuclear bodies called PML oncogenic domains (PODs) (17). PODs are also known as ND10 (21) or Kr bodies (16). PML is believed to colocalize with the autoantigens in primary biliary cirrhosis, but the two proteins are not directly known to interact with each other (16, 17, 22). It is speculated that PML first interacts with other unknown protein factors and then is indirectly associated with the autoantigens. This type of PML-associated protein factor has yet to be identified. In APL cells induced to differentiated by ATRA, the abnormal microspeckled PML pattern can be reorganized to the normal PODs (16, 17). In RA-resistant, APL-derived NB4 cells, POD formation cannot be induced by ATRA treatment (17). Together, these...
results indicate that the ability of PML to form PODs is required for the induction of differentiation in APL cells. Taking advantage of the fact that PML can suppress the formation of NIH3T3 cell foci by activated neu (18, 19), we have constructed several PML mutants and examined their ability to form PODs in vivo and suppress transformation. Here, we demonstrate that PML mutants lacking the nuclear localization signal, the dimerization domain, and the RING finger domain are unable to form PODs and that their ability to suppress foci formation is significantly reduced. However, mutants lacking the proline-rich domain at the N-terminal and the serine-proline-rich domain on the C-terminal can form PODs in vivo and can suppress foci formation, much like the wild-type PML. Our results corroborate the previous finding that the ability of APL cells to reassemble PODs after ATRA treatment is an important event in differentiation induction (17). Furthermore, we show that PMLprb- PMLNls-, and PMLprb-nls- are potential dominant negative inhibitors of PML and that the RING finger motif may interact with other nuclear proteins.

MATERIALS AND METHODS

Plasmid Construction—The PML expression plasmid pSG5PML, as described in our previous report (18), was used to construct PML deletion mutants. Mutant PMLNls-—see Fig. 1) was created by digesting pSG5PML with MluI and Smal, blunt ended with a Klenow fragment, and circularized with T4-DNA ligase. Mutant PMLprb—was constructed by digesting pSG5PML with MluI and BamHI and then blunt ended and circularized with T4-DNA ligase. Mutant PMLdim—was constructed by completely digesting pSG5PML with KpnI, then ethanol precipitated, resuspended in high stringency S1 buffer (200 mM NaCl, 50 mM sodium acetate, pH 4.5, 1 mM ZnSO4, 0.5% glycerol), and treated with 3000 units of S1 nuclease (Life Technologies, Inc.) at 37°C for 1 h. The resulting linear plasmid was further treated with a Klenow fragment and circularized with T4-DNA ligase. Clones with an in-frame deletion were identified by direct DNA sequencing and retained. A clone lacking PML nucleotides 684–888 (B), which encodes the entire putative leucine zipper, was designated PMLdim—. Deletion mutants PMLpr- PMLpr-—were constructed by polymerase chain reaction amplification of pFM211 (the original cDNA clone used to construct pSG5PML (18)) using the following primers: MU1, 5'-AAGGATCCACATGAGGAGGATTTCACT-3'; MU2, 5'-GAGGATCCACATTGATTCCTTCACTTAC-3'; MU3, 5'-GAAGGATCCACATGAGGAGGATTTCACTTAC-3'; MU4, 5'-GAAGGATCCACATGAGGAGGATTTCACTTAC-3'. The major features of the PML protein (Fig. 1) include the proline-rich domain at the N-terminal, which resembles the transactivating domain of some transcription factors (7, 20, 26). A RING finger motif, B-box-1, and B-box-2 lie immediately downstream. Other important domains include the a-helical domain responsible for dimerization, a nuclear localization signal, and a proline-serine-rich domain at the C-terminal believed to be involved in PML phosphorylation. In each mutant PML constructed by us as shown in Fig. 1 and as described under “Materials and Methods,” the deleted region was confirmed by direct DNA sequencing. For mutants PMLpr- PMLpr-- and PMLprb-, the 5'-ends of these clones were sequenced and the presence of the correct ATG translation start site and the perfect Kozak's sequence was confirmed in each clone.

To confirm that these mutant clones would translate their respective mutant PML proteins, each of these plasmids was transfected into NIH3T3 cells by lipofectamine-mediated gene transfer. Western blot analysis of protein fractions isolated from these cells indicated that PMLdim-- and PMLNls-- encoded mutant PML proteins of the expected size (approximately 80–90 kDa) (Fig. 2, lanes 6 and 7). However, mutant PMLpr-- PMLpr-- and PMLprb- encoded mutant proteins significantly smaller than expected. We have previously reported that although its predicted size is about 72 kDa, the PML protein migrates at about 90 kDa during SDS-polyacryl-
amidine gel electrophoresis, probably due to its acidic nature
(18). As shown in Fig. 2A, lane 2, PMLapro— with a deletion of
the first 48 amino acids migrated at about 70 kDa, indicating
that is much smaller than the wild-type PML. To confirm that
there is no additional deletion within the cDNA sequence, we
completely sequenced one strand of PMLapro— and found no
deletion in any other region of the cDNA. We speculate that the
significant difference in size between PMLapro— and PML is the
result of a change in secondary structure. The proline-rich
domain may be responsible for the slower running nature of the
wild-type PML in SDS-polyacrylamide gel electrophoresis. This
notion is supported by the fact that both PMLpro— and PML-
prb— migrated at molecular weights agreeable with their pre-
dicted sizes (Fig. 2A, lanes 3–5).

To further confirm these findings, the PML mutant plasmids
were transcribed and translated in vitro. Fig. 2B showed that
the molecular weights of the in vitro transcribed and translated
PMLapro—, PMLpro—, PMLprb—, and PMLdim— proteins agree
with those derived from the Western blotting analysis as shown
in Fig. 2A.

Cellular Localization of PML Mutant Proteins—Since PML
had been shown to concentrate within PODs (16, 17, 22, 25), we
intended to define which domains of PML are required for the
formation of PODs. How deleting various domains of PML
affects its cellular localization was determined by transfecting
the PML mutants into NIH3T3 cells. The PML mutant proteins
were visualized in the cell by immunofluorescence staining
using the affinity-purified PML antibody as described in our
previous reports (18, 19). As shown in Fig. 3, d and h, PMLpro—
and PMLsp— did not affect the cellular location of PML. A
typical nuclear speckled pattern made up of PODs was found in
cells transfected with these plasmids. Deletion of the region
harboring the nuclear localization signal resulted in cytoplasmic
and perinuclear localization (Fig. 3b), presumably because the
PML mutant protein could not enter the nucleus. A similar
pattern of cellular distribution of PMLdim—, PMLpr—, and
PMLprb— was found in NIH3T3 cells transfected with these
plasmids. A diffuse instead of speckled nuclear pattern was
found (Fig. 3, c, e, and f). The double mutant (PMLprb—nls—),
unlike PMLnls—, was diffused throughout the cytoplasm (Fig.
3g). Since only an additional deletion of the RING finger motif
in PMLprb—nls—, it is suggested that the RING finger motif
may be responsible for interaction with other protein factors
and that its deletion disrupts the perinuclear pattern. Toget-
er, the above results indicate that the dimerization domain,
the RING finger domains, and the nuclear localization
signal are all required for the organization of PODs, since
deletion of these regions resulted in diffuse nuclear distribu-
tion and the inability of PMLs to assemble into POD.

Effect of PML Mutants on Suppression of Transformation of
NIH3T3 Cells by Activated neu—In our previous report (19),
we demonstrated that PML suppresses neu-transformed NIH3T3
cells by suppressing both the expression of neu and its signal-
ing events. To investigate the ability of these PML mutants to
suppress transformation of NIH3T3 cells, each individual mut-
ant was cotransfected with c-Nau104 (genomic neu) by calcium
phosphate coprecipitation as described under “Materials and
Methods.”

Results of these studies (Fig. 4) showed that PMLdim—,
PMLnls—, PMLpr—, PMLprb—, and PMLprb—nls— lost their
ability to suppress transformation of NIH3T3 by activated neu.
This suggests that the dimerization domain, nuclear localiza-
tion signal, and RING finger motif are all required for the PML
growth suppressor function. As shown in Fig. 3, all of these PML mutants share one common property, i.e. they are unable to assemble into a nuclear speckled pattern or the PODs. This suggests that the ability of PMLs to organize into PODs is required for PML to exert its growth or transformation suppressor function. This notion is supported by the finding that PML mutants PMLpro– and PMLsp–, which are capable of forming PODs (Fig. 3, a and d), were both able to suppress neu-induced transformation of NIH3T3 cells, just like the wild-type PML (Fig. 4).

Fig. 4. The effect of mutant PMLs on suppression of neu-induced transformation of NIH3T3 cells. Cotransfection of plasmids by calcium phosphate coprecipitation and focus-forming assays were performed as described previously (18). In each transfection assay, 10 μg of plasmid was transfected. The results shown represent means of three independent experiments with three plates each. In each transfection, 2 μg of p5v-Migal (β-galactosidase cDNA under the control of the SV40-early promoter) was included, and the activity of β-galactosidase was determined to monitor transfection efficiency. Plasmid c-neu104 containing the activated neu oncogene has been described previously (18); PML and its mutants used in the study are described in this manuscript, Borden et al. (7) that PML proteins bearing a mutation in the cysteine-rich (RING finger) motif (Glu<sup>59</sup>Cys<sup>60</sup>→ Glu<sup>59</sup>Leu<sup>60</sup>) had a diffuse nuclear staining pattern. During the preparation of this manuscript, Borden et al. (29) also demonstrated by site-directed mutagenesis that the RING finger motif of PML is...

Fig. 5. Cellular localization patterns of PML in human fibroblast GM637D cells. The GM637D cells were transfected with 2–3 μg of the PML mutant plasmid constructs by lipofectamine (Life Technologies, Inc.). After 48 h of culture, immunofluorescence staining was performed as described previously (19); a, wild-type PML; b, PMLprb–; c, double mutant (PMLprb–nls–); d, PMLnls–. Magnification, ×1000.

DISCUSSION

To understand the function of PML, a growth suppressor involved in the t(15;17) translocation in APL, we constructed several mutant plasmids and used them to analyze its functional domains. We found that (i) the nuclear localization signal, dimerization domain, and RING finger motif of PML are all essential for the formation of PODs; (ii) the ability of PML to form PODs in vivo is required for its growth suppressor function; and (iii) PMLprb–, PMLnls–, and PMLprb–nls– are potential candidates for dominant negative inhibition of the wild-type PML. Moreover, we have uncovered evidence that the RING finger motif of PML may interact with other proteins.
and during normal or pathological proliferative states, indicating PML increased significantly in the inflammatory tissues genesis. Interestingly, Terris implicated not only in APL but also in non-hematological oncology, confirmed by another group, who found that when skin, breast, 21, 22, 30, 31). Activation of both cellular and viral genes in infection) (16, 17, 21, 22, 30). These proteins, however, do not contain any of the proteins known to be involved in pre-mRNA splicing, transcription, and DNA synthesis (17, 30). In addition to PML, the PODs may include the SP100, NDP-55, and Vmw110 proteins and an unidentified 65-kDa protein (16, 17, 22, 30). These proteins, however, do not interact directly with PML. In studies of APL and herpes simplex virus immediate-early protein, PODs appeared to be extremely important (both directly and indirectly) for myeloid differentiation as well as regulation of gene expression (e.g., activation of both cellular and viral genes in infection) (16, 17, 21, 22, 30, 31).

Recently, the growth suppressor function of PML was confirmed by another group, who found that when skin, breast, and colon malignant cells turned invasive, PML expression was lost (32). These data imply that PML may be an oncogene involved not only in APL but also in non-hematological oncogenesis. Interestingly, Terris et al. (33) reported that expression of PML increased significantly in the inflammatory tissues and during normal or pathological proliferative states, indicating that PML plays a role in the inflammatory process and in cell growth control.

As stated above, the PML-RARα fusion protein is reportedly a dominant negative inhibitor of PML and RXR (7, 17, 34). Previous immunofluorescence staining of PML indicated cytoplasmic and/or nuclear localization with a micropunctuated pattern in APL blasts and in cells stably transfected with PML-RARα. Here, we have demonstrated that PMLprb, PMLnls, and PMLprb–nls are potential dominant negative inhibitors against the normal function of PML and that transient expression of PMLprb, PMLnls, and PMLprb–nls significantly reduces or completely eliminates normal PODs in GM637D cells. Furthermore, we have shown that PMLprb, PMLnls, and PMLprb–nls can block the effect of wild-type PML on transformation of NIH3T3 cells by activated neu. We have noticed that stable transfecitants overexpressing PMLnls in GM637D cells eventually died (data not shown). Since overexpression of PML in B104–1-1 (19) and HeLa cells2 did not result in cell death, we therefore reason that PMLnls probably induced the observed cell death through its perinuclear localization. It is possible that the RING finger motif interacts with other important unknown proteins essential for cell growth. Sequestration of these proteins by PMLnls may be responsible for the cell death. Therefore, PMLnls may not be a suitable dominant negative inhibitor. Conversely, PMLprb and PMLprb–nls, both without the RING finger motif, should be more suitable for testing the effect of knocking out wild-type PML in normal cells and for studying whether elimination of cellular PML will result in growth stimulation. We plan to establish in our laboratory stable transfecitants of GM637D constitutively expressing the PMLprb and PMLprb–nls and to investigate the effects of these dominant negative inhibitors on proliferation and growth of GM637D cells. Such studies will be helpful in further understanding the function of PML in the control of cell growth.

As stated above, PML is characterized by the RING finger motif. This motif has the sequence C1X2C2X(9–27)C3X(1–3)XH1–X2–C4–X5–C6–X14–48–C6–X2–C2, where C represents cysteine, H represents histidine, X can be any amino acid, and the numbers in parentheses refer to the length in amino acids of the sequences (20, 27). The RING finger motif may play an important cellular role by acting as a DNA binding or protein–protein interacting domain (9, 20, 29). It is necessary then to determine whether DNA or protein is involved in the interaction with the RING finger motif of PML protein. The fact that treatment of the nuclei with RNase and DNase in previous studies did not disrupt the PODs in human cell lines suggests that nucleic acids are not a component of this structure (30). Consequently, this implies that it is the RING finger motif that normally interacts with protein factors. Our results presented in Figs. 3 and 4 show that the immunostaining pattern of mutant PMLnls is clearly distinct from that of double mutant PMLprb–nls and that PMLnls– accumulated in the cytoplasm, mostly in the perinucleus. In contrast, the staining pattern of the double mutant was fine and diffuse throughout the cytoplasm. This suggests that the RING finger motif accounts for these differences. We therefore speculate that the RING finger motif of PML interacts with other nuclear protein factors and is responsible for the differences in cellular distribution between PMLnls– and PMLprb–nls–.

We conclude then that the RING finger motif, α-helical region, and nuclear localization signal of PML are all required for the formation of PODs. We also conclude that the ability of

2 X.-F. Le, P. Yang, and K.-S. Chang, unpublished results.
3 X.-F. Le, P. Yang, and K.-S. Chang, unpublished observations.
PML to form PODs is essential for its growth and transformation suppressor function. However, studies are needed (i) to define more precisely the specific contribution of each domain in PML and (ii) to define the composition and function of PODs so as to understand in depth their role during human oncogenesis. In this direction, we have been assessing the effects of the two dominant negative inhibitors of wild-type PML described in this report (PMLprb and PMLprb-nls) on the proliferation and differentiation of GM637D cells, which stably express mutant PMLs.

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