Cancer Cell
Article

RXR Is an Essential Component of the Oncogenic PML/RARA Complex In Vivo

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DOI 10.1016/j.ccr.2007.06.004

SUMMARY

Although PML-enforced RARA homodimerization allows PML/RARA to bind DNA independently of its coreceptor RXR, the latter was identified within the PML/RARA complex. We demonstrate that a PML/RARA mutant defective for RXR binding fails to trigger APL development in transgenic mice, although it still transforms primary hematopoietic progenitors ex vivo. RXR enhances PML/RARA binding to DNA and is required for rexinoid-induced APL differentiation. In RA-treated PML/RARA-transformed cells, the absence of RXR binding results in monocytic, rather than granulocytic, differentiation. PML/RARA enhances posttranslational modifications of RXRA, including its sumoylation, suggesting that PML-bound sumoylation enzymes target RXRA and possibly other PML/RARA-bound chromatin proteins, further contributing to deregulated transcription. Thus, unexpectedly, RXR contributes to several critical aspects of in vivo transformation.

INTRODUCTION

Acute promyelocytic leukemia (APL) is caused by the expression in hematopoietic cells of the PML/RARA oncogene. This fusion protein is a potent transcriptional repressor that interferes with gene expression programs involved in both progenitor self-renewal and terminal myeloid cell differentiation (Sell, 2005). It was proposed that PML/RARA homodimerization, through a coiled coil in PML, is key to transformation, and indeed, forced RARA dimerization recapitulates many properties of PML/RARA, including corepressor/HDAC recruitment and target gene silencing (Grignani et al., 1998; Kwok et al., 2006; Licht, 2006; Lin and Evans, 2000; Lin et al., 1998; Minucci et al., 2000; Sternsdorf et al., 2006; Zhou et al., 2006). Recent studies have also demonstrated that specific posttranslational modifications of PML/RARA, such as sumoylation, are essential for transformation (Zhu et al., 2005). While the ability of the fusion protein to negatively regulate transcription appears to be essential for transformation, treatment with retinoic acid (RA) induces complete remissions (Warrell et al., 1993), at least in part through reactivation of PML/RARA-dependent transcription.

The normal RARA receptor can only bind DNA when coupled to another class of nuclear receptor, the RXRs, which are the promiscuous partners of many different class II nuclear receptors (Kastner et al., 1995). Within these heterodimeric complexes, RXRs can contribute to repression or activation (Calleja et al., 2006; Kuriyan, 2004), in part through specific posttranslational modifications,

SIGNIFICANCE

Acute promyelocytic leukemia (APL) is caused by translocations always involving a transcription factor, RARA. Several studies have stressed the importance of translocation-induced RARA dimerization in the transformation process. Here, we demonstrate that the presence of the RARA heterodimeric partner RXR in the PML/RARA complex is required for leukemogenesis in transgenic mice. RXR greatly facilitates the binding of PML/RARA to DNA, but titration of RXR by PML/RARA could also contribute to transformation. Drugs that activate RXR relieve PML/RARA-induced transcriptional repression and trigger APL differentiation, only when RXR is PML/RARA bound. Finally, in PML/RARA-expressing cells, RXR undergoes a specific modification, sumoylation, which could further enhance transcriptional repression. Thus, through several distinct mechanisms, RXR is an unsuspected essential factor in APL pathogenesis.

such as phosphorylation or sumoylation (Choi et al., 2006; Gianni et al., 2003; Mann et al., 2005; Srinivas et al., 2005; Tarrade et al., 2005; Zimmerman et al., 2006). While early experiments had demonstrated that PML/RARA can bind RXR (Perez et al., 1993), the actual presence of RXR within the DNA-bound PML/RARA oncogenic complex was only demonstrated recently (Kamashev et al., 2004). RXR facilitates the binding of PML/RARA to widely spaced direct repeats (Kamashev et al., 2004), a property that is important for transformation ex vivo (Zhou et al., 2006). When coupled to activation of cAMP signaling, RXR-specific ligands (rexinoids) were shown to differentiate APL cells, as well as many non-APL myeloid human leukemia samples (Altucci et al., 2005; Benoit et al., 1999). In APL cells, this association in fact activates transcription of PML/RARA-specific target genes, unifying the mechanisms of APL cell differentiation (Kamashev et al., 2004).

We have investigated the role of RXR in the PML/RARA complex through a variety of ex vivo and in vivo experiments and demonstrate that, while RXR is dispensable for immortalization of primary hematopoietic progenitors ex vivo, it is absolutely required for APL development in transgenic mice. The presence of RXR in the PML/RARA complex not only facilitates DNA binding, but is also required for rexinoid-induced differentiation, demonstrating that RXR is not a silent partner, but a critical determinant of transformation.

RESULTS

Using structural information from the crystallized RARA/RXR heterodimer, we had previously identified two positions in RARA (M379 and T382) that are essential for RXR binding (Zhu et al., 1999). While in the context of RARA this mutation abrogates DNA binding, PML-enforced dimerization allows the formation of a PML/RARA*M883R,T886R* homodimers/DNA complex, confirming that PML/RAR binding to DNA does not require RXR (Figure 1A) (Jansen et al., 1995; Perez et al., 1993). The RXR interaction domain of RARA is embedded within the ligand-binding domain and could potentially interfere with ligand-dependent activation. To demonstrate that PML/RARA*M883R,T886R* remains a RA-dependent transcriptional activator, we evaluated by quantitative RT-PCR the transcriptional response to RA of PML/RARA-specific genes, such as *tgII* (Benedetti et al., 1996; Zhou et al., 2006), in primary hematopoietic progenitor cells transduced with PML/RARA or PML/RARA*M883R,T886R* (Figure 1B). In contrast to mock-infected cells, a clear induction of *tgII* expression was observed in response to RA administration for both fusion proteins, establishing that this point mutation does not affect ligand-dependent transcriptional activation. We also questioned whether PML/RARA*M883R,T886R* might be degraded upon exposure to RA, a feature of DNA-binding and transcriptionally active RARs (Zhu et al., 1999). PML/RARA*M883R,T886R*, in sharp contrast to the parental RARA*M379R,T382R*, was efficiently degraded upon RA exposure (Figure 1C). Note that the sumoylation pattern of the PML/RARA mutant was identical to that of PML/RARA (Figure 1C) and that sumoylation remained arsenic sensitive (data not shown), demonstrating that the inability to bind RXR does not affect this critical posttranslational modification (Lallemand-Breitenbach et al., 2001; Zhu et al., 2005).

Enhanced binding of SMRT onto PML/RARA homodimers was proposed to be the critical event leading to transcriptional repression of RA target genes and leukemogenesis (Kwok et al., 2006; Lin and Evans, 2000; Minucci et al., 2000; Sternsdorf et al., 2006; Zhou et al., 2006). Whether the presence of RXR in this complex affects SMRT binding or its stability upon RA exposure was never examined. The PML/RARA//RXR complex was reproducibly more sensitive to RA-induced SMRT dissociation than the PML/RARA homodimeric complex (Figure 1D). Indeed, in the presence of RXR, SMRT was completely dissociated from the complex at a concentration of 200 nM, while the PML/RARA homodimer retained a significant SMRT binding, which was maintained even with RA concentrations greater than 1 μM, consistent with previous studies (Lin and Evans, 2000; Minucci et al., 2000; Figure 1D, lower panel; and data not shown). Thus, the RA dose response of SMRT dissociation from the PML/RARA//RXR heterotetramer closely resembles the RA dose response of APL cell differentiation (Quenech'Du et al., 1998).

We then questioned whether RXR binding would be dispensable for PML/RARA-induced transformation of primary hematopoietic progenitors ex vivo (Du et al., 1999). The fusion protein defective for RXR binding induced a sharp differentiation arrest and allowed indefinite replating of the colonies (Figure 2A). Yet, subtle differences were noted between the cells transformed by these two fusion proteins, as untreated PML/RARA*M883R,T886R*-transformed cells had more monocytic features on May Grunwald Giemsa (MGG) stains or fluorescence-activated cell sorting (FACS) analysis (greater expression of the Mac1 monocytic antigen than Gr-1, a granulocytic differentiation marker), when grown in methylcellulose or in liquid cultures (Figure 2B). PML/RARA*M883R,T886R* was usually expressed at 2- to 3-fold higher levels than the parental fusion protein (Figure 1C). We examined whether PML/RARA*M883R,T886R*-transformed cells would retain RA sensitivity. In methylcellulose, a dramatic response to RA was manifested by a sharp decrease in the number of dense colonies, identical to the one found in PML/RARA-transformed cells (Figure 2B). Interestingly, after 8 days in methylcellulose in the presence of RA, PML/RARA-transformed cells yielded granulocytes, while PML/RARA*M883R,T886R*-transformed ones differentiated into monocytes, as assessed by both MGG staining and expression of the myeloid antigens Mac1 and Gr-1 (Figure 2B). Similarly, in liquid culture, PML/RARA-transformed cells differentiated into both monocytes and granulocytes, while PML/RARA*M883R,T886R*-transformed cells showed only monocytic features upon RA treatment. This unexpected monocytic differentiation was observed with three different transductions, including one where

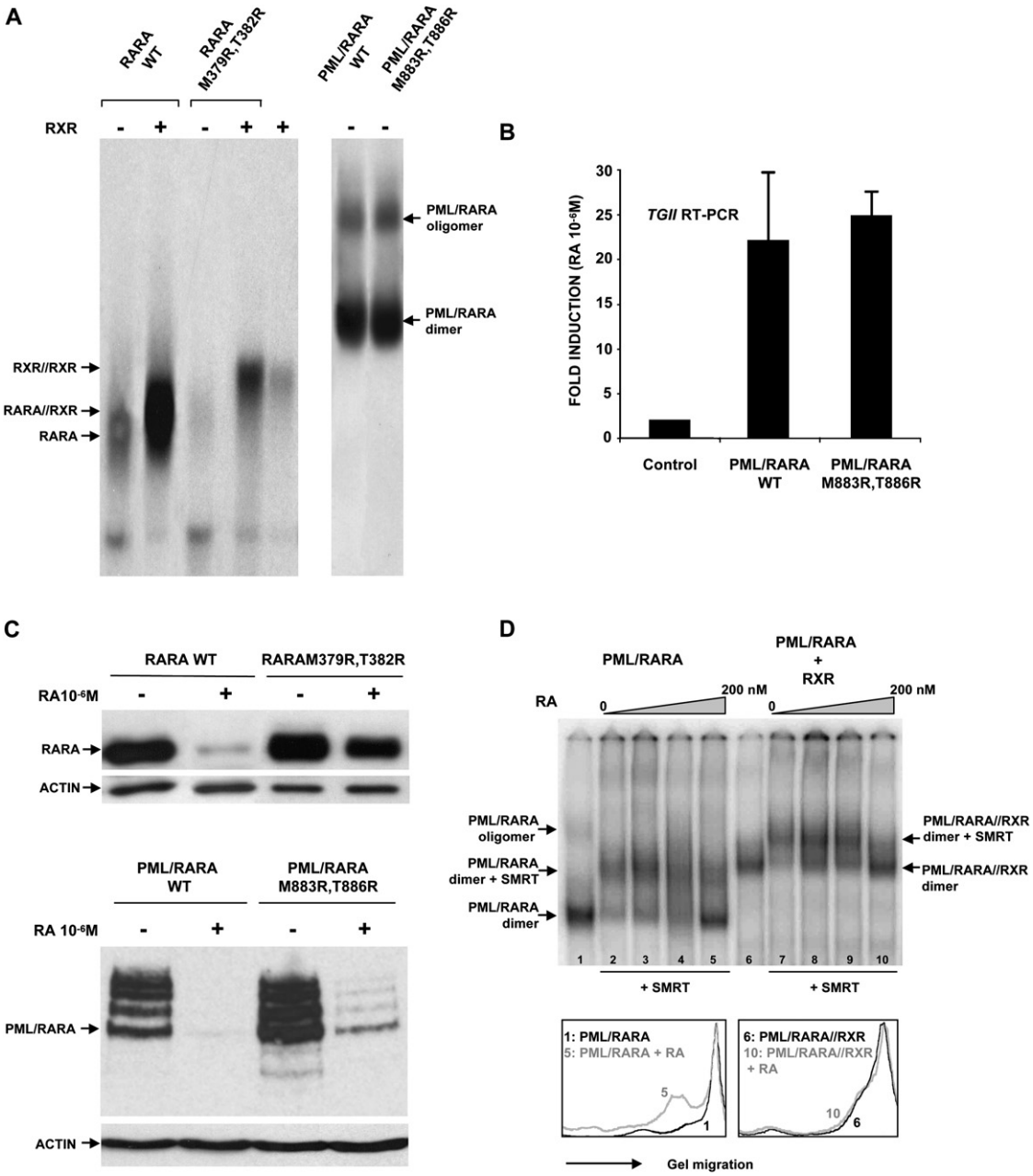

Figure 1. Functional Characterization of a PML/RARA Mutant Defective for RXR Binding

(A) Gel shift analysis with a DR5 RARE of different RARA derivatives with or without RXR, as indicated.

(B) Quantitative PCR analysis of a PML/RARA-specific gene, *tgII* (Benedetti et al., 1996), in primary hematopoietic progenitor cells transduced by PML/RARA or PML/RARA*M883R,T886R* after a 15 hr exposure to 10^{-6} M RA. Means ± standard deviation (SD).

(C) (Upper panel) Cos cells transiently transfected with RARA or RARA*M379R,T382R*, treated overnight with 10^{-6} M RA. (Lower panel) Cells analyzed in (B), with or without retinoic acid. Note that defective RXR binding does not affect the sumoylation pattern of PML/RARA.

(D) RXR modulates binding of SMRT to the PML/RARA DNA complex. (Lower panel) Quantifications of the critical lanes (1, 5, 6, and 10) of the autoradiogram. Note that 200 nM RA restores the baseline profile of the PML/RARA//RXR complex, but not of the PML/RARA complex.

the levels of expression of the wild-type or mutant fusions were similar.

RXR is normally a transcriptionally silent partner of the RXR/RAR heterodimer, due to RXR subordination (Germain et al., 2002). Yet, the rexinoid/cAMP association was shown to induce a robust transcriptional activation of RA target genes, such as *RARB*, through desubordination of the transcriptional activation domain of RXR (Altucci et al., 2005; Kamashev et al., 2004). Interestingly, in the context of cytokine-treated cells, in methylcellulose or in liquid cultures, rexinoids triggered some differentiation of PML/RARA-transformed cells, while cAMP on itself did not,

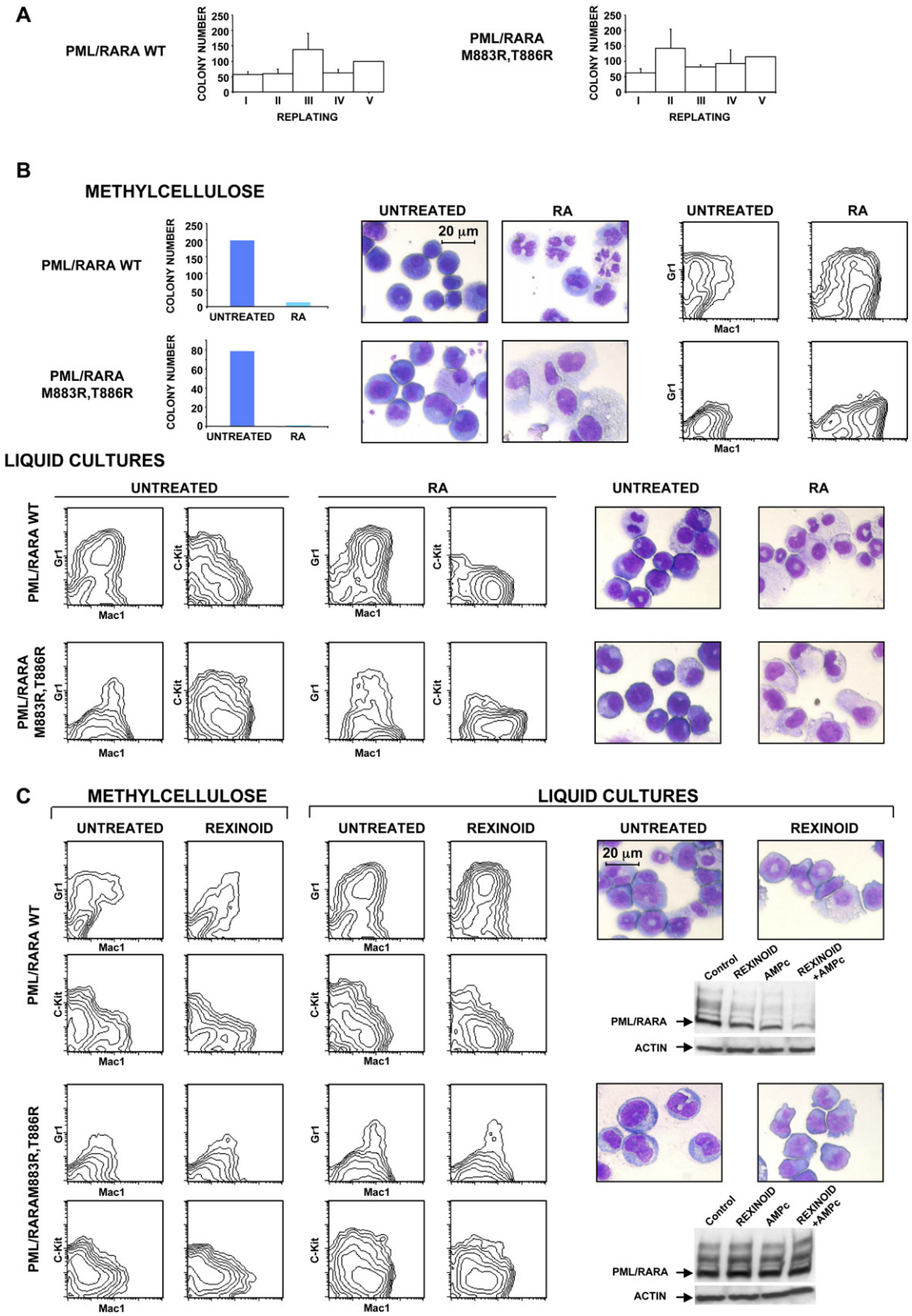

Figure 2. Role of RXR Binding in PML/RARA-Induced Transformation and Response to RA or Rexinoids

(A) Transduction of PML/RARA, or its RXR-binding-defective mutant, both transform primary hematopoietic progenitors. Replating assay in methylcellulose. Means ± standard deviation (SD).

and the rexinoid/cAMP association was identical to rexinoids alone (Figure 2C and data not shown). Rexinoid-triggered differentiation was not as complete than the one triggered by RA and was not associated with a decrease in colony numbers (data not shown). It was essentially manifested by a reproducible decrease in Kit expression, with an increase in Mac1 staining and some morphological changes toward greater maturity (Figure 2C). The rexinoid/cAMP association led to the synergistic degradation of PML/RARA, but not PML/RARA*M883R,T886R* (Figure 2C), suggesting that, in that setting, transcriptional derepression by rexinoids contributes to the differentiation process and that full PML/RARA degradation is not a prerequisite for differentiation. Importantly, PML/RARA*M883R,T886R*-transformed cells were completely unresponsive to rexinoids and also failed to degrade the fusion protein upon exposure to the association of rexinoid and cAMP. Finally, as expected, addition of rexinoids to retinoids yielded additive effects (whether cAMP was present or not), as previously noted in other transformed myeloid cells (Shiohara et al., 1999), and this only in cells transformed by PML/RARA, but not its mutant (data not shown). Altogether, while RXR binding to PML/RARA is dispensable for ex vivo transformation, it modulates the phenotype of the transformed cells and is required for response to rexinoids.

To further address the importance of RXR binding by PML/RARA in the leukemogenic process in vivo, we generated PML/RARA*M883R,T886R* transgenic mice under the transcriptional control of the MRP8 promoter (Brown et al., 1997; Zhu et al., 2005). Eight founders were obtained, which all expressed PML/RARA (Figures 3A, 3D, and 3E). Serial analyses of the peripheral blood demonstrated an increase in the proportion of normal myeloid elements in six of the eight lines (Figure 3B). Enlargement of the spleen was repeatedly noted in 30% of the founders or offspring, with the disappearance of the normal lymphoid follicles. This was accompanied by a very dense bone marrow, with a clear predominance of myeloid cells in MGG staining or FACS analysis of marrow aspirates (Figures 3C and 3F). Rare proliferations of hematopoietic cells were found in the liver. These myeloproliferative diseases were transplantable with a low efficiency and a long latency. Strikingly, not a single APL was observed, despite a more than 3 years follow-up of founders, their offspring, or their engrafted marrows into irradiated syngeneic recipients (Figure 3A). At large, the levels of PML/RARA*M883R,T886R* expression in the bone marrow were slightly higher than those previously observed in PML/RARA transgenics (Zhu et al., 2005), despite significant overlaps (Figure 3E and data not shown). Note that, in contrast to cathepsin G promoter PML/RARA transgenic, with the promoter used here, higher levels of PML/RARA expression are usually associated with a higher incidence of APL (Westervelt et al., 2003; Zhu et al., 2005). Expression of the fusion protein in marrows of PML/RARA*M883R,T886R* transgenics were lower than in retrovirally transduced progenitor cells (Figure 3E, left part). In keeping with the observations in methylcellulose cultures, several transgenics expressing high levels of PML/RARA*M883R,T886R* protein (lines 14, 44) had a clear excess of monocytes in the peripheral blood (Figure 3B) and in the bone marrow (Figure 3F). Interestingly, MRP8-PML/RARA transgenics exhibit a complex skin disease (Brown et al., 1997; Hansen et al., 2003; Zhu et al., 2005). The severity of these lesions is directly proportional to PML/RARA expression and independent of its K160 residue (S. Kogan, personal communication, and Zhu et al., 2005). Intriguingly, none of the founder mice or their offspring ever exhibited any type of skin lesion (Figure 3G). Altogether, PML/RARA*M883R,T886R* transgenic mice only develop a mild myeloproliferative syndrome (Kogan et al., 2002) that never evolves toward full-blown APL.

Many of the features of PML/RARA*M883R,T886R* described so far could result from a lower affinity for its DNA-binding sites. In the absence of RXR, both PML/RARA or PML/RARA*M883R,T886R* bound DR5, DR8, or DR12 response elements (Figures 1A and 4A) (Kamashev et al., 2004). Coexpression of RXR dramatically increased the binding efficiency of PML/RARA, but not of PML/RARA*M883R,T886R* to all three elements (Figure 4A). To directly investigate the consequence of this difference in DNA-binding affinity observed in gel shift analysis, mouse embryo fibroblasts (MEFs) in which the three RARs were excised and in which any RA-induced transcriptional modification is dependent on the transduced receptor (Altucci et al., 2005) were transduced by PML/RARA or PML/RARA*M883R,T886R*. Despite a higher expression of the mutant fusion by western blot (Figure 4B), only PML/RARA reproducibly activated the expression of two (*rarb* and *tgII*) of the three primary target genes tested (*rarb*, *cyp26A*, and *tgII*), PML/RARA*M883R,T886R* being completely inactive in this assay (Figure 4B and data not shown). Contrarily to the prevailing model, PML/RARA expression did not induce significant changes in the baseline levels of these three target genes (data not shown). That the levels of the two fusion proteins in fibroblasts were lower than those in transduced hematopoietic cells may explain why *tgII* activation was observed in one system (Figure 1B), but not the other (Figure 4B). An alternative explanation could be that the corepressor/coactivator content of those two types of cells is very different or that the epigenetic chromatin environment of the PML/RARA binding site(s) within the *tgII* promoter significantly differs between MEFs and transformed primary hematopoietic progenitors. In that respect, while *tgII* may be induced several hundred folds in APL cells, its amplitude of induction in MEFs is only 7-fold (Benedetti et al., 1996; Zhou et al., 2006). Finally, in transient transfections where PML/RARA blunts the transcriptional response of a

(B) Response to RA (10^{-6} M) treatment for a week in methylcellulose or 3 days in liquid culture of transduced cells at passage 3.
(C) Response to rexinoids (BMS 649, 3 μM) in the same conditions as above. (Right) Western blot analysis after an overnight treatment with rexinoids and/or cAMP.

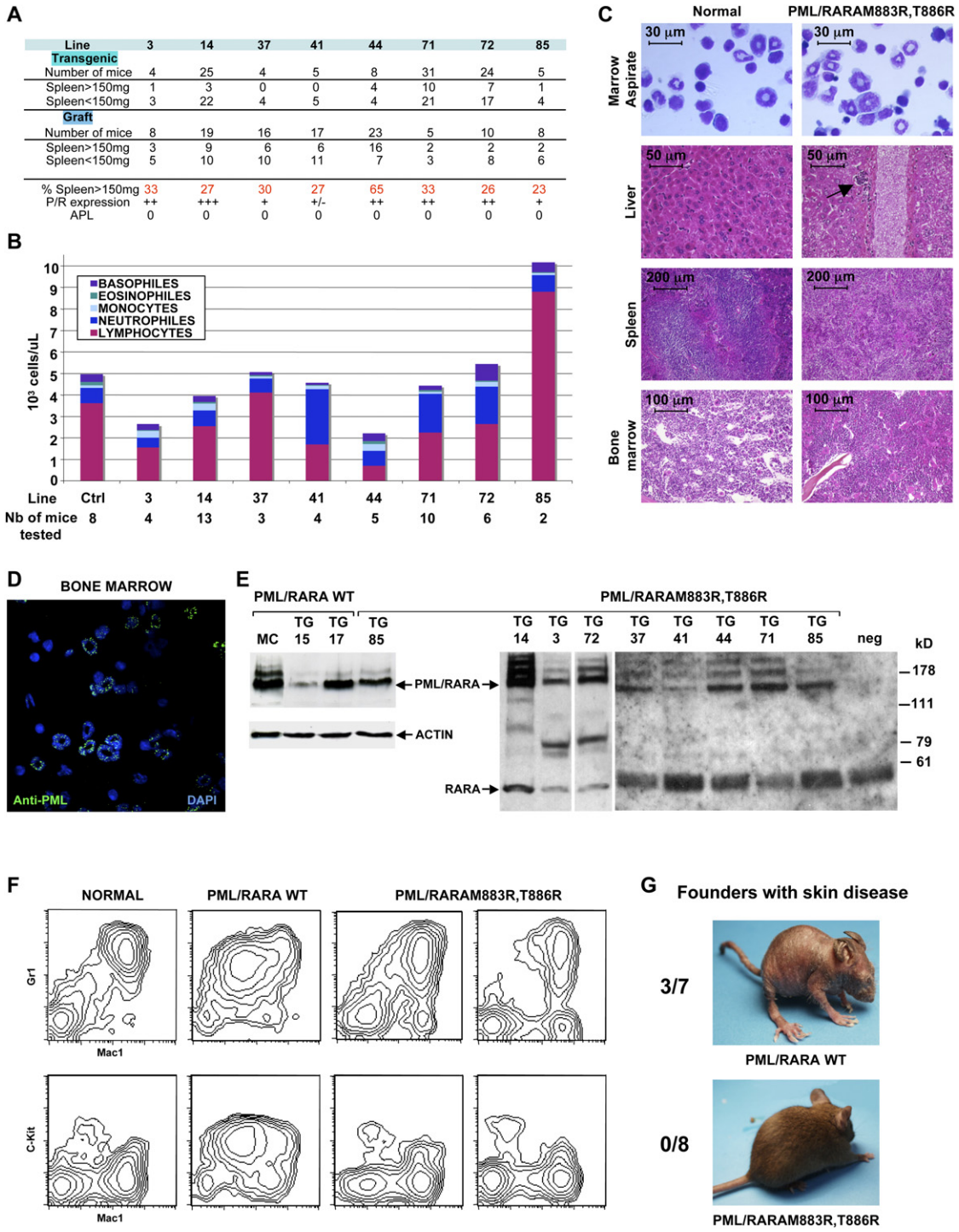

Figure 3. PML/RARA*M883R,T886R* Transgenic Mice Do Not Develop APL

(A) Summary of the mouse data. Transgenics (founders or offspring) that developed a large spleen are indicated, as well as primary grafts of transgenic bone marrow. None of these mice ever developed APL.

(B) Mean values of blood count from the indicated number of mice in each line.

(C) Histologic features of a normal mice and a representative PML/RARA*M883R,T886R* with spleen enlargement. Note the very dense bone marrow and the destructured architecture of the spleen.

(D) Immunofluorescence of transgenic bone marrow demonstrating high PML/RARA expression in granulocytes.

(E) Western blot analysis of bone marrow extracts from mice (founders or offspring) in the different transgenic lines. (Left panel) Comparison between PML/RARA-transduced hematopoietic progenitors (MC), two representative PML/RARA transgenic (PR) (Zhu et al., 2005), and the

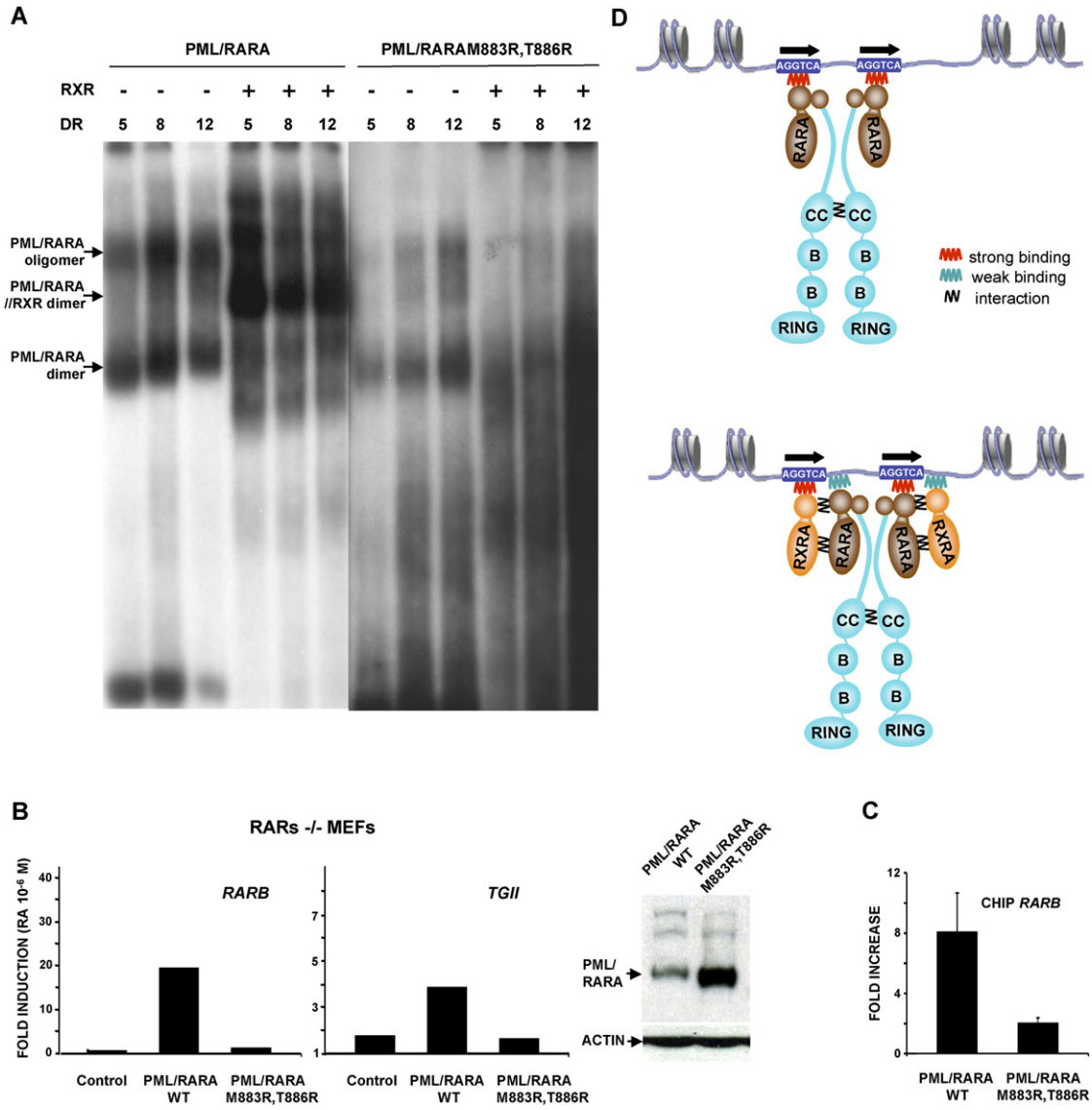

Figure 4. RXR Binding Increases the DNA-Binding Affinity of PML/RARA

(A) Gel shift analysis. Binding of the normal or mutant fusion protein, with or without RXR, to various AGGTCA direct repeat (DR) response elements (Kamashev et al., 2004) is shown.

(B) (Left panel) quantitative RT-PCR analysis of RA target genes in *RARs*$^{-/-}$ MEFs stably transduced by the indicated fusion proteins and treated overnight with 10^{-6} M RA (mean of two independent transductions). Western blot analysis of these cells is shown in the right panel.

(C) ChIP analysis of PML/RARA or PML/RARA*M883R,T886R* binding to the cloned *RARB* promoter in transfected Cos cells, expressed as increase over nonspecific binding. Means ± standard deviation (SD).

(D) Model for the enhancing effect of RXR binding on PML/RARA DNA-binding properties. Strong interactions are noted by a red broken line, while weak ones are indicated by a green one.

RA-sensitive reporter gene (RARE-3-Tk-Luc) (de Thé et al., 1991), the mutant was much less potent than the parental fusion in blocking RA response (data not shown). To directly demonstrate that in vivo PML/RARA*M883R,T886R* binds less efficiently to response elements in a cellular context, we performed a chromatin immunoprecipitation (ChIP) experiment. Cos cells were transfected with the *RARB* promoter, which contains a well-characterized RA-responsive sequence (de Thé et al., 1990) activated by PML/RARA (Zhou et al., 2006; Figure 4C), together with

PML/RARA*M883R,T886R* transgenic line 85. (Right panel) All eight lines of PML/RARA*M883R,T886R* transgenics and a littermate negative control. A PML/RARA cleavage product is occasionally observed (Lane and Ley, 2003).

(F) Flow cytometry analyses of bone marrows from normal, APL, and two distinct PML/RARA*M883R,T886R* mice with myeloproliferative disease.

(G) In contrast to PML/RARA transgenic mice (Zhu et al., 2005), PML/RARA*M883R,T886R* mice never develop a skin defect.

expression vectors for PML/RARA or PML/RARA*M883R,T886R* and RXR. Identical levels of RXR and the fusion proteins were obtained (data not shown). After immunoprecipitation of the PML/RARA-DNA complex and quantification of the precipitated *RARB* sequence by quantitative PCR, 4-fold higher levels of DNA were consistently recovered with PML/RARA compared to PML/RARA*M883R,T886R* (Figure 4C). Although of potential interest, ChIP analysis of endogenous PML/RARA target genes was not attempted in primary bone marrow cells because of the very low level of PML/RARA expression and the low affinity of PML or RARA antibodies. Altogether, these studies strongly support the idea that the greater DNA-binding efficiencies of PML/RARA//RXR heterotetramers, compared to PML/RARA homodimer, contribute to a more potent gene regulation (Figure 4D).

We then questioned whether RXR binding onto PML/RARA could result in changes in RXR stability or posttranslational modifications. Indeed, RXRs may be phosphorylated, ubiquitinylated, or sumoylated, phosphorylation occurring upon RA exposure or MAPK or JNK activation (Choi et al., 2006; Gianni et al., 2003; Mann et al., 2005; Srinivas et al., 2005; Takano et al., 2004; Zimmerman et al., 2006). We first compared the expression of RXRA, the major RXR isoform expressed in myeloid cells (Taschner et al., 2007), in primary hematopoietic progenitors transformed by PML/RARA or PML/RARA*M883R,T886R*. A striking increase in the abundance of low-mobility RXRA-reactive protein species was noted, in PML/RARA-transformed cells only, while RXRA expression was often moderately increased. In every experimental system tested (primary hematopoietic progenitors, U937 cells, retrovirally transduced MEFs or NB4 cells), PML/RARA triggered a 3- to 10-fold increase in the abundance of this modified RXRA protein (Figure 5A and data not shown). Similarly, in transfected cells, PML/RARA stabilized RXRA (moderately in HeLa and massively in CHO cells) and further enhanced the abundance of this low-mobility form (Figure 5B, upper part; and data not shown). No quantitative or qualitative change in RXRB expression was noted upon PML/RARA expression (data not shown).

PML or PML/RARA are extensively sumoylated proteins (Lallemand-Breitenbach et al., 2001; Muller et al., 1998; Zhu et al., 2005). The sumoylation machinery recruited onto the PML moiety of PML/RARA (Lallemand-Breitenbach et al., 2001; Zhu et al., 2005) might also enhance sumoylation of PML/RARA-bound RXRA. In transfected HeLa or CHO cells, coexpression of sumo1, but not sumo2, increased the abundance of the low-mobility RXRA-reactive protein, an effect dramatically enhanced in the presence of PML/RARA, (Figure 5B, upper panel). These observations suggest that the slow-migrating form of RXRA is an RXRA-sumo conjugate, although an initial transient sumoylation could trigger a subsequent ubiquitination or phosphorylation (Huang et al., 2003; Ulrich, 2005). We then transiently expressed PML/RARA together with RXRA, Ubc9, YFP-sumo1, or YFP-sumo2 in HeLa cells and immunoprecipitated RIPA-soluble proteins. Expression of YFP-sumo1 and, to a lesser extent, YFP-sumo2, yielded novel RXRA-reactive proteins (Figure 5B, bottom panel), which were immunoprecipitated with anti-RXRA or GFP antibodies (Choi et al., 2006) and whose abundance was reproducibly increased by PML/RARA expression (Figure 5B, bottom panel). In this experiment, PML/RARA did not induce significant RXRA stabilization, allowing significant comparison of the sumoylated RXRA species' abundance. These experiments directly demonstrate that PML/RARA enhances RXRA sumoylation.

We finally investigated which domains of PML/RARA were required for enhancement of RXRA modification. To this end, we expressed in both primary hematopoietic cells or MEFs a number of PML/RARA mutants (Figure 5C and data not shown). The arsenic-induced K160 sumoylation site in PML (Lallemand-Breitenbach et al., 2001; Zhu et al., 2005) was not only dispensable for RXRA sumoylation but actually reproducibly enhanced it (Figure 5C and data not shown). In contrast, disrupting the PML RING finger in PML/RARA (C88SP89R) abrogated the enhanced modification of RXRA, while a point mutation in the RA-binding domain of the RARA moiety of PML/RARA (Shao et al., 1997) did not. As expected, overexpression of RARA did not change the baseline modification of RXRA (Figure 5C). Altogether, PML/RARA-enhanced RXRA modification requires tethering onto RXR of the PML functional domains required for PML sumoylation (Duprez et al., 1999; Kamitani et al., 1998; Lallemand-Breitenbach et al., 2001).

DISCUSSION

Here we demonstrate the essential role of RXR in PML/RARA-induced APL leukemogenesis and outline some of the mechanisms involved. While PML/RARA may bind DNA in the absence of RXR, the heterotetramer formed in its presence has a much greater DNA-binding affinity (Figures 4A and 4D), which likely accounts for its more potent biological properties. At the molecular level, the presence of four DNA binding sites that all recognize the AGGTCA core motif likely explains the cooperative binding ex vivo or in vivo, as the DNA-binding domains not interacting with the specific AG(G/T)TCA half-sites may also contribute to the stability of the complex through non-sequence-specific interactions with DNA. We had previously demonstrated that the binding of PML/RARA to very widely spaced half-sites, which is required for transformation (Zhou et al., 2006), was greatly favored in the presence of RXR (Kamashev et al., 2004). Finally, DR1 response elements are only recognized by the PML/RARA//RXR heterotetramer, but not by PML/RARA homodimers, presumably because of steric hindrance (Jansen et al., 1995; Kamashev et al., 2004; Perez et al., 1993). Hence, reduced DNA-binding affinity or a more restricted binding site repertoire likely plays a critical role in the weaker phenotype of the mutant.

The greater biological effects of PML/RARA, when compared to its defective mutant, does not reflect a higher level of protein expression, as PML/RARA*M883R,T886R* was usually expressed at higher levels than the parental

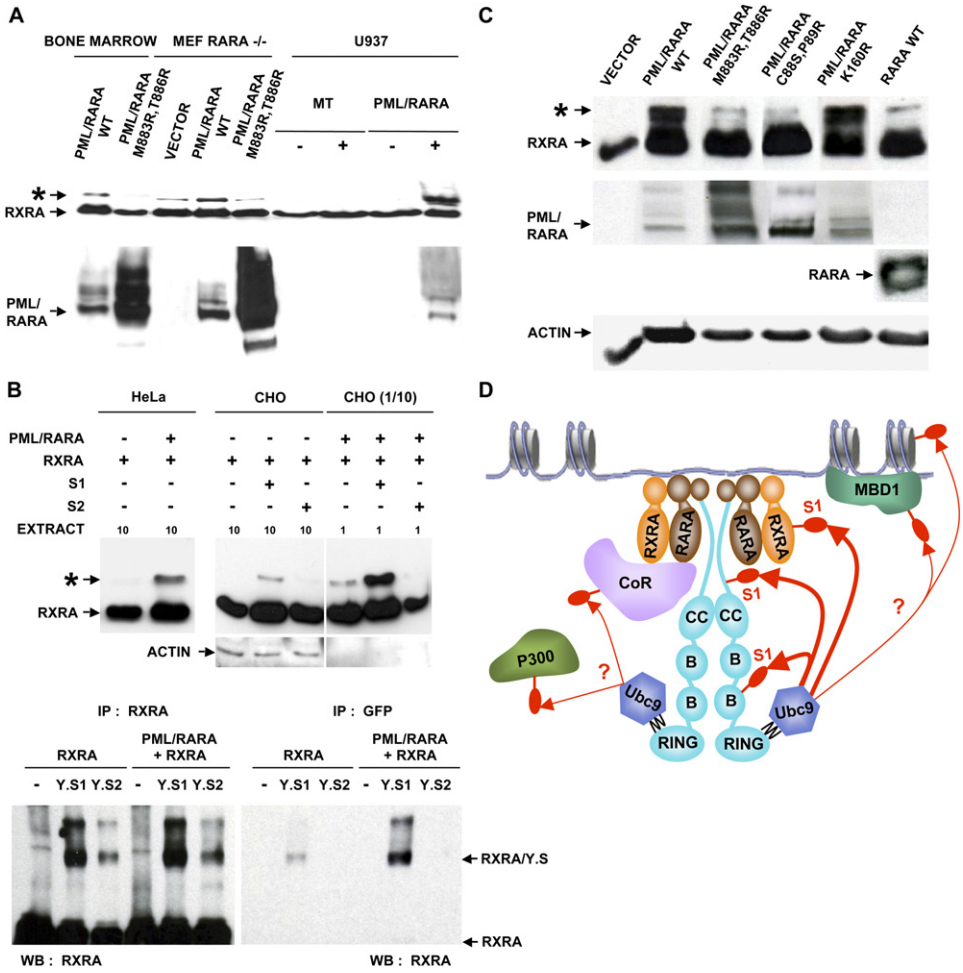

Figure 5. PML/RARA Enhances Posttranslational Modifications of RXRA

(A) Whole-cell extracts from immortalized bone marrow progenitors, MEFs or U937 cells (Grignani et al., 1993), were probed with an anti-RXRA (upper panel) or RARA (lower panel). The amounts of extracts loaded were adjusted for similar RXRA expression. The modified RXR species are indicated by an asterisk. The addition of zinc to induce PML/RARA expression in U937 cells is indicated by + or −; MT refers to the metallothionein promoter control.

(B) HeLa or CHO cells were transfected with the indicated constructs and probed for RXRA expression. (Upper panel) PML/RARA expression stabilizes RXRA and increases its modified form (asterisk). Different amounts of whole-cell extracts were analyzed to allow comparison of the efficiency of RXRA modification. (Bottom panel) PML/RARA enhances conjugation of RXRA to YFPsumo1. RIPA extracts of transfected HeLa cells were immunoprecipitated with anti-RXRA (left) or anti-GFP (right). RXRA and YFP-sumo/RXRA are indicated.

(C) RXRA posttranslational modification (asterisk) in primary hematopoietic progenitors transduced with the indicated PML/RARA mutants.

(D) Model of PML/RARA-mediated tethering of the PML-bound sumoylation machinery onto the chromatin environment.

fusion. Moreover, the pattern of posttranslational modifications, such as sumoylation, was identical between the parental and the mutant protein (Figure 1C). We favor the idea that the greater expression of the mutant fusion could result from both its reduced toxicity and/or its greater stability. Both of these features may reflect its lower affinity for DNA, as DNA binding was shown to be a critical determinant of RARA degradation (Zhu et al., 1999). PML/RARA is an extremely toxic protein in fibroblasts (Lavau et al., 1996), and in our hands, PML/RARA expression remained very low whatever the multiplicity of infection used, while that of PML/RARA*M883R,T886R* could reach 50 times that of the parental protein with repeated courses of retroviral infection (data not shown). Note, however, that in PML/RARA transgenics very similar levels of expression were obtained (Figure 3E), allowing a direct and relevant comparison of the leukemogenic properties of the wild-type and mutant protein in vivo.

The consequences of the RXR-binding mutation were different in the two biological systems analyzed here. While both PML/RARA and its mutant efficiently transformed hematopoietic progenitors ex vivo, PML/RARA*M883R,T886R* transgenic mice developed a mild nonfatal myeloproliferative disease, which did not transplant well, but never APL. This discrepancy is not unprecedented: RARA was shown to immortalize primary hematopoietic progenitors (Du et al., 1999), while transgenic mice expressing several types of dominant-negative RARA

never developed APL (Matsushita et al., 2006). Similarly, several self-dimerizing RARA were shown to efficiently promote transformation ex vivo, while this process is very inefficient in transgenic mice (Kwok et al., 2006; Sternsdorf et al., 2006; Zhou et al., 2006; Zhu et al., 2005). Thus, the ex vivo and in vivo models of PML/RARA-induced transformation do not have identical genetic requirement.

RXR-dependent signaling regulates many important aspects of hematopoiesis (Johnson et al., 1999; Rusten et al., 1996; Taschner et al., 2007). PML/RARA-triggered transformation was suggested to involve RXR titration away from its other partners (Kastner et al., 1992; Perez et al., 1993). Note that, in the context of RARA-triggered ex vivo transformation (Du et al., 1999), mutation in the DNA-binding domain abolished ex vivo transformation (data not shown), implying that titration of RXR is not the sole basis for the phenotype, as suggested in other systems (Kastner et al., 1995; Saitou et al., 1995). Our approach, in contrast to those using extinction of RXR expression, allows a direct assessment of the consequences of RXR titration by PML/RARA. Ex vivo and to a lesser extent in vivo, RXR binding by the fusion protein modulates the phenotype of undifferentiated or RA-differentiated cells: PML/RARA*M883R,T886R*-transformed cells had monocytic features and differentiated into typical monocytes (Figure 2B; Figures 3B and 3F). Interestingly, in normal myeloid cells, extinction of RXRA expression is required for granulocytic differentiation, while conversely, enforced RXRA expression favored monocytic differentiation (Taschner et al., 2007), suggesting that RXRA titration/degradation by PML/RARA could contribute to the granulocytic differentiation of RA-treated APL cells. Similarly, the skin phenotype of PML/RARA transgenics, which bears many similarities with that of *RXRs*$^{-/-}$ mice (Li et al., 2000), was never observed in any of the PML/RARA*M883R,T886R* transgenics (Figure 3G), even in lines with high transgene expression and myeloproliferative disease. Altogether, these observations argue for a contribution of RXR titration to PML/RARA-dependent events, including RA-induced differentiation.

Recent evidence has demonstrated that RXR is not a transcriptionally silent partner within the RXR/RARA or PML/RARA//RXR complexes. In fact, upon dissociation of SMRT from RARA, induced by cAMP-triggered PKA activation, rexinoids can become potent transcriptional activators of RARA or PML/RARA target genes and trigger APL cell differentiation (Altucci et al., 2005; Benoit et al., 1999; Kamashev et al., 2004). That RXR binding to PML/RARA is required for rexinoid-induced differentiation is fully consistent with our previous observations. Unexpectedly, however, the rexinoid/cAMP combination induced neither full differentiation nor growth arrest in PML/RARA-transformed cells in methylcellulose assays, liquid culture, or APL mice (Guillemin et al., 2002) (Figure 2; data not shown). As rexinoids were only shown to trigger terminal differentiation in human cells (Altucci et al., 2005; Benoit et al., 1999), mouse RXRA could be a weaker activator than its human counterpart.

We demonstrate that PML/RARA promotes posttranslational modifications of RXRA, including sumoylation, which triggers transcriptional repression for RXRA as for many other transcription factors (Choi et al., 2006; Hay, 2005; Verger et al., 2003). We cannot rule out that RXRA binding onto PML/RARA does not also promote other modifications, such as phosphorylations, which trigger RXRA transcriptional activation, nuclear export, and proteasome-dependent degradation (Gianni et al., 2003; Srinivas et al., 2005; Zimmerman et al., 2006). Yet, arsenic-induced RXRA phosphorylation (Mann et al., 2005; Tarrade et al., 2005) yielded a distinct pattern of modification (data not shown). That PML/RARA can enhance RXRA sumoylation suggests that fusion of PML to RARA tethers an active sumoylation machinery in the vicinity of PML/RARA chromatin-binding sites (Figure 5D), conceivably modifying not only RXRA, but also neighboring chromatin proteins such as histones, corepressors, and coactivators, whose functions are tightly regulated by sumoylation (Hay, 2005). Our observations thus raise the prospect that dysregulated sumoylation induced by PML/RARA may contribute to altered gene expression and contribute to APL pathogenesis. Unfortunately, this hypothesis cannot be directly tested ex vivo, because RXR binding to PML/RARA is dispensable in that setting. RING finger mutations that impair RXRA sumoylation (Figure 5C) also affect sumoylation of PML/RARA K160 (Kamitani et al., 1998; and data not shown), which is critical for ex vivo and in vivo transformation (Zhu et al., 2005). More elaborate strategies using in vivo substitution of RXRA by a nonsumoylatable mutant would be required to establish the in vivo importance of RXRA sumoylation in APL pathogenesis. Altogether, RXR facilitates PML/RARA-induced transformation through several different mechanisms but also participates in the differentiation response, identifying this coreceptor as an unsuspected key member of the oncogenic complex.

EXPERIMENTAL PROCEDURES

Retroviral Transductions

The cDNA encoding *PML/RARA* corresponds to the bcr1 breakpoint and includes the alternatively spliced exon 5 as well as the PML nls (de Thé et al., 1991). The two mutations that abolish RXR binding to RARA were transferred from RARA*M379R/T382R* into PML/RARA (Zhu et al., 1999). Infection of lineage-depleted bone marrow from 5-fluorouracil-treated C57BL/6 mice, culture, and G418 selection of the transduced progenitors cells in methylcellulose with stem cell factor, IL3, IL6, and GM-CSF were performed exactly as previously described (Du et al., 1999). After a week, neomycin-selected cells were recovered from methylcellulose and either analyzed (FACS, MGG staining, immunofluorescence, and western blot) or replated at a density of 10,000 cells per dish. Cells were serially replated until they stopped growing. Cell growth in liquid culture was assessed in RPMI 1640 medium supplemented with IL3, IL6, and SCF.

Protein Analyses

Electrophoretic shift analyses were performed as previously described (Kamashev et al., 2004; Zhu et al., 1999), using extracts from Cos-transfected cells, bacterially produced SMRT, and the DR5 RA response element (RARE) from the *RARB* gene (de Thé et al., 1990) or canonical response elements with various spacings, as previously

described (Kamashev et al., 2004). When analyzing the effect of RA on the interaction between the SMRT corepressor and the different PML/RARA complexes, RA treatment was performed directly in the transfected cells used to prepare the extracts. Western blotting was performed using an antibody RP115 kindly provided by P. Chambon. Anti-RXRA were from Santa-Cruz, while anti-RXRB were from Abcam.

ChIPs were performed in CHO or Cos cells transfected with 100 ng of a 5 kb fragment of the *RARB* promoter (de Thé et al., 1990) and 50 ng of PSG5-RXRA and PSG5-PML/RARA or -PML/RARA*M883R,T886R*. Immunoprecipitation was then performed using an Active Motif ChIP kit and a cocktail of anti-PML monoclonals. Quantitative PCR was performed on the rescued DNA using primers encompassing the RARE and a specific probe (available upon request). Results were expressed as fold induction over background in the absence of PML/RARA expression. Immunoprecipitations were performed as previously described (Zhu et al., 1999), using anti-RXRA or anti-GFP antibodies.

Immortalized *RARs*$^{-/-}$ MEFs (Altucci et al., 2005) were infected by retroviruses expressing various PML/RARA derivatives, as described before (Zhou et al., 2006). Quantitative RT-PCR was performed using Light-cycler technology (Roche) and commercially available primers and probes from the *rarb*, *cyp26a*, and *tgII* genes (Applied Biosystems). Rexinoids (BMS 649) were used at a concentration of 3 μM. 8-(4-chlorophenylthio)adenosine 3-5′ cyclic monophosphate (8-CPT-cAMP) was used at a concentration of 2.10^{-4} M.

Transgenic Mice

PML/RARA was expressed using the MRP8 promoter (Brown et al., 1997) in B6/CBA F1 hybrids. Mice were kept under SPF conditions and used under protocols approved by the Institutional Animal Care and Use Committee (CREEA4). Hematological disorders, such as splenomegaly, were never observed in nontransgenic littermates. Eight PML/RARA*M883R,T886R* transgenic lines expressing the fusion proteins by both western blot and immunofluorescence were obtained. Myeloproliferation was defined by the association of splenomegaly (spleen larger than 150 mg) with highly hyperplastic marrow, in the absence of significant differentiation abnormalities (Kogan et al., 2002). F0 founders were sacrificed, and phenotypes were observed in offspring (when available) or primary grafts in irradiated syngeneic recipients. Blood cell counts were performed with a Celldyn 3700 counter (Abbott Diagnostic). Bone marrow and pathology analyses were performed as before (Lallemand-Breitenbach et al., 1999).

ACKNOWLEDGMENTS

This work was supported by the INCa/Canceropole, ARECA, P2R, ATIP, National Natural Science Foundation of China (30525006), and EPITRON, an Integrated Project funded by the European Union under the 6th Framework Programme (LSHC-CT-2005-518417). R.N. was supported by the E. Lilly and later the Lady TATA foundations; J. Zhou was supported by the French ministry for Foreign Affairs and VCP. We warmly thank S. Kogan for the MRP8 promoter; H. Gronemeyer for BMS compounds; P.G. Pelicci for Zn-inducible U937 cells; D. Morello and P. Mercier for generating the transgenic mice in UMR 5547 CNRS/UPS, IFR 109; M. Lebras and V. Lallemand-Breitenbach for helpful discussions; M. Pla, head of the mouse facility, for assistance; A. Desveaux, L. Legrès, and all technicians of the pathology department for efficient help; M.T. Daniel and A. Janin for help with slide interpretation; and the IPSEN Foundation for providing the environment where part of this manuscript was written. We warmly thank D. Moras, IGBMC, Strasbourg, France for identifying the two RARA residues that abrogate RXR binding.

Received: December 15, 2006
Revised: April 4, 2007
Accepted: June 1, 2007
Published: July 9, 2007

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