Forced retinoic acid receptor α homodimers prime mice for APL-like leukemia

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Summary

***RARA* becomes an acute promyelocytic leukemia (APL) oncogene by fusion with any of five translocation partners. Unlike RARα, the fusion proteins homodimerize, which may be central to oncogenic activation. This model was tested by replacing PML with dimerization domains from p50NFκB (p50-RARα) or the rapamycin-sensitive dimerizing peptide of FKBP12 (F3-RARα). The X-RARα fusions recapitulated in vitro activities of PML-RARα. For F3-RARα, these properties were rapamycin sensitive. Although in vivo the artificial fusions alone are poor initiators of leukemia, p50-RARα readily cooperates with an activated mutant CDw131 to induce APL-like disease. These results demonstrate that the dimerization interface of RARα fusion partners is a critical element in APL pathogenesis while pointing to other features of PML for enhancing penetrance and progression.**

Introduction

Recurring chromosomal translocations are a hallmark of many leukemias and are often associated with a specific leukemic phenotype (Rabbitts, 1994). In the case of acute promyelocytic leukemia (APL), all known disease-associated translocations involve the retinoic acid receptor α (*RARA*) locus on chromosome 17, leading to the expression of leukemia-specific RARα fusion proteins (Melnick and Licht, 1999). Although RARα normally requires the heterodimer partner retinoid X receptor (RXR) to bind DNA, the X-RARα fusion proteins overcome this dependence and can efficiently bind target sequences as homodimers (de Thé et al., 1990; Dyck et al., 1994; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991).

Whereas five different N-terminal sequences in the fusion proteins have been described, they all retain identical C-terminal portions of RARα, supporting the proposal that the receptor DNA binding domain (DBD) and ligand binding domain (LBD) are important in the development of leukemia. In more than 99% of cases, RARα becomes fused to PML in a t(15;17) reciprocal translocation (Redner, 2002). PML is a RING finger protein of the Trim/RBCC family. Special attention was initially given to the fact that PML-RARα disrupts the normal localization of the PML protein in PML oncogenic domains (PODs, also called PML bodies), which are macromolecular proteinaceous complexes in the nucleoplasm. This process is reversed by retinoic acid treatment, which also triggers differentiation of the leukemic cells (Dyck et al., 1994; Koken et al., 1994). The rarely detected alternate fusion partners PLZF [t(11;17)], Nucleophosmin [t(5;17)], NuMA [t(11;17)], and STAT5b [(der17)], however, do not affect PML domains. They do not share any obvious functional or structural homologies, except for the presence of a dimerization/oligomerization domain that is retained in the fusion protein. In previous studies, we (Lin and Evans, 2000) and others (Minucci et al., 2000) have shown that the PML-derived oligomerization domain of PML-RARα is essential for its biological properties. Forced oligomerization of RARα can lead to similar biological characteristics in vitro, such as enhanced binding of

SIGNIFICANCE

In acute promyelocytic leukemia (APL), retinoic acid receptor α (RARα), which functions as a heterodimer with the retinoid X receptor (RXR), is converted to an oncoprotein by fusion to various partners (X-RARα) but most frequently to the PML protein. X-RARα fusions interact with RXR but can also homodimerize, conferring expanded binding site specificity and suggesting that homodimerization may be linked to oncogenic activation. We addressed this proposal by demonstrating that forced dimerization is sufficient for most in vitro activities of X-RARα and can promote leukemogenesis in vivo. These results suggest that interference with spontaneous dimerization of fusion proteins may provide a strategy for therapeutic intervention in acute myeloid leukemia.

the corepressors SMRT and N-CoR, and blocking differentiation of myeloid cell lines.

The diversity of fusion partners and their implicated involvement in apoptosis pathways (PML, PLZF, Nucleophosmin) has created a controversy as to the exact extent of their contribution—in particular that of the PML protein—to leukemogenesis. We have addressed this question by creating RARα fusions to two heterologous oligomerization domains, which were then expressed in the hematopoietic compartment in mice. Since neither of the two artificial fusion proteins carries PML sequences or affects the PML bodies, they represent suitable tools to dissect the contribution of the RARα and PML pathways for leukemogenesis, respectively.

The data obtained in this study demonstrate that artificial X-RARα fusions recapitulate many of the activities of natural PML-RARα oncoprotein in vitro, and that such activity is dependent on dimerization. Although RARα dimers are poor inducers of leukemia on their own when expressed in vivo, they can prime mice for development of leukemia. Breeding of the p50-RARα transgene into a *Pml* null background did not lead to increased leukemia incidence, arguing that rather than simply having a dominant-negative effect over RARα and PML pathways, PML-RARα is a gain-of-function fusion.

Results

Design of X-RARα fusion proteins

One common feature of all RARα fusion proteins is the presence of a homodimerization/homooligomerization domain. In order to dissect this property from other biological activities, we fused the common RARα part to two different heterologous dimerization domains. In these X-RARα fusion proteins, the RARα part is identical to that in the naturally occurring PML-RARα, PLZF-RARα, NPM-RARα, NuMA-RARα, and STAT5b-RARα fusions. In the first construct, we used the dimerization domain of the NFκB p50 subunit (p50-RARα) (described previously in Lin and Evans, 2000). In the second, we used the FK506 binding protein (12 kDa) (FKBP12) (Amara et al., 1997). For FKBP12, we took advantage of a constitutively dimerizing mutant ($FKBP12^{F36M}$) (Rollins et al., 2000). Dimerization of this mutant is abrogated in the presence of its ligand rapamycin, and when fused in a reiterated manner, this protein creates a dimerization interface that can be rapidly and reversibly regulated by rapamycin. We created a head-to tail $FKBP12^{F36M}$ trimer fused to RARα (F3-RARα). Figure 1A depicts a schematic overview of the proteins used in this study.

Artificial X-RARα fusion proteins do not disrupt PML nuclear bodies

We sought to separate abnormal dimerization of RARα from other putative activities of proteins that are fused to RARα in APL. Specifically, we wished to assess whether the PML domain of PML-RARα contributed to leukemogenesis through disruption of nuclear bodies, homodimerization, or both, or, possibly, through a novel activity. In principle, the artificial X-RARα fusion proteins should help to specifically delineate the importance of homodimerization. We therefore examined whether these proteins had an effect on endogenous PML distribution. To this end, we used an MSCV-based retroviral vector to express p50-RARα, F3-RARα, and as a control, PML-RARα in murine ECoM-G cells (Sykes and Kamps, 2001). At 24 hr postinfection,

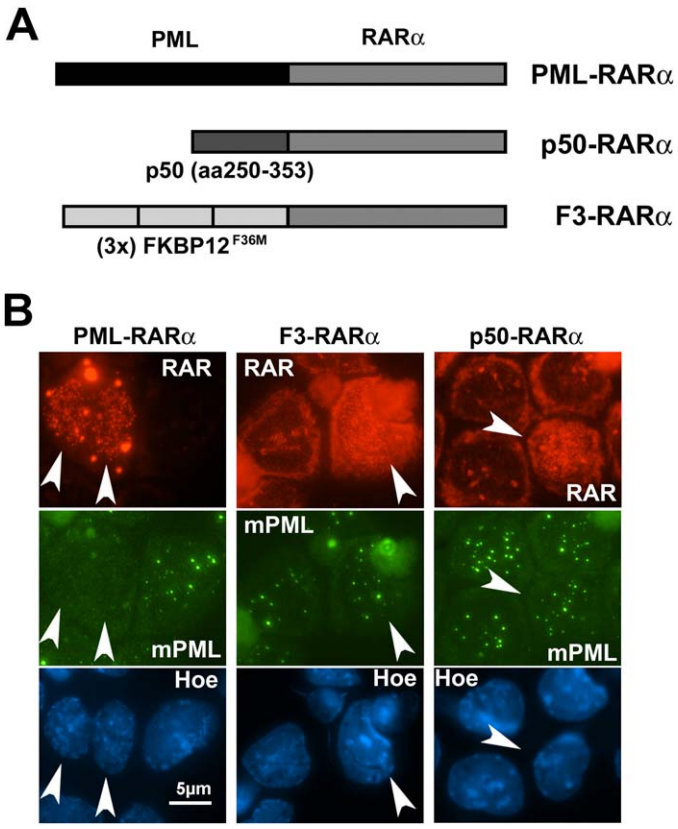

Figure 1. X-RARα fusion proteins: Structure and effects on PML nuclear bodies

A: Schematic representation of X-RARα fusion proteins used in the study. RARα sequences were fused to the dimerization domain of the NFκB p50 subunit (p50 aa 250–353), or to three head-to-tail copies of the constitutively dimerizing mutant F36M of the rapamycin binding protein FKBP12.

B: p50-RARα or F3-RARα proteins do not disrupt PML nuclear bodies. Murine myeloid ECoM-G cells were infected with retroviral constructs expressing PML-RARα, F3-RARα, or p50-RARα, respectively. Double immunofluorescence analysis was carried out with a monoclonal antibody (mAb) specific for murine PML (middle panels, FITC) and a rabbit antiserum against RARα (top panels, Texas red). PML-RARα, but not p50-RARα or F3-RARα, disrupts the endogenous PML pattern. Human PML (and PML-RARα) is not recognized by the mAb against murine PML (left, middle panel). The bottom panels show the corresponding cells in the blue channel stained by Hoechst 33258 to visualize nuclei.

we performed a cytospin preparation of the cells followed by immunostaining. A monoclonal antibody (mAb) specific for murine PML (Figure 1B, middle panels, FITC, green), together with a rabbit antiserum specific for RARα, was used to detect transduced X-RAR proteins (Figure 1B, top panels, Texas red) for double immunofluorescence staining. The anti-mouse PML mAb does not crossreact with human PML and is therefore unreactive with our transduced PML-RARα. PML-RARα, p50-RARα, and F3-RARα exhibit nuclear as well as some cytoplasmic staining. Nuclear PML-RARα exhibits the expected microspeckled pattern (top left panel), and F3-RARα and p50-RARα show a similar granular nuclear localization (top, middle, and right panels, respectively; expressing cells are marked by arrows). When stained for endogenous mouse PML, PML-RARα-expressing cells (arrows) exhibit a disrupted, almost invisible pattern, whereas neither F3-RARα-expressing cells nor p50-RARα-expressing cells had altered localization of PML. The bottom panels show the

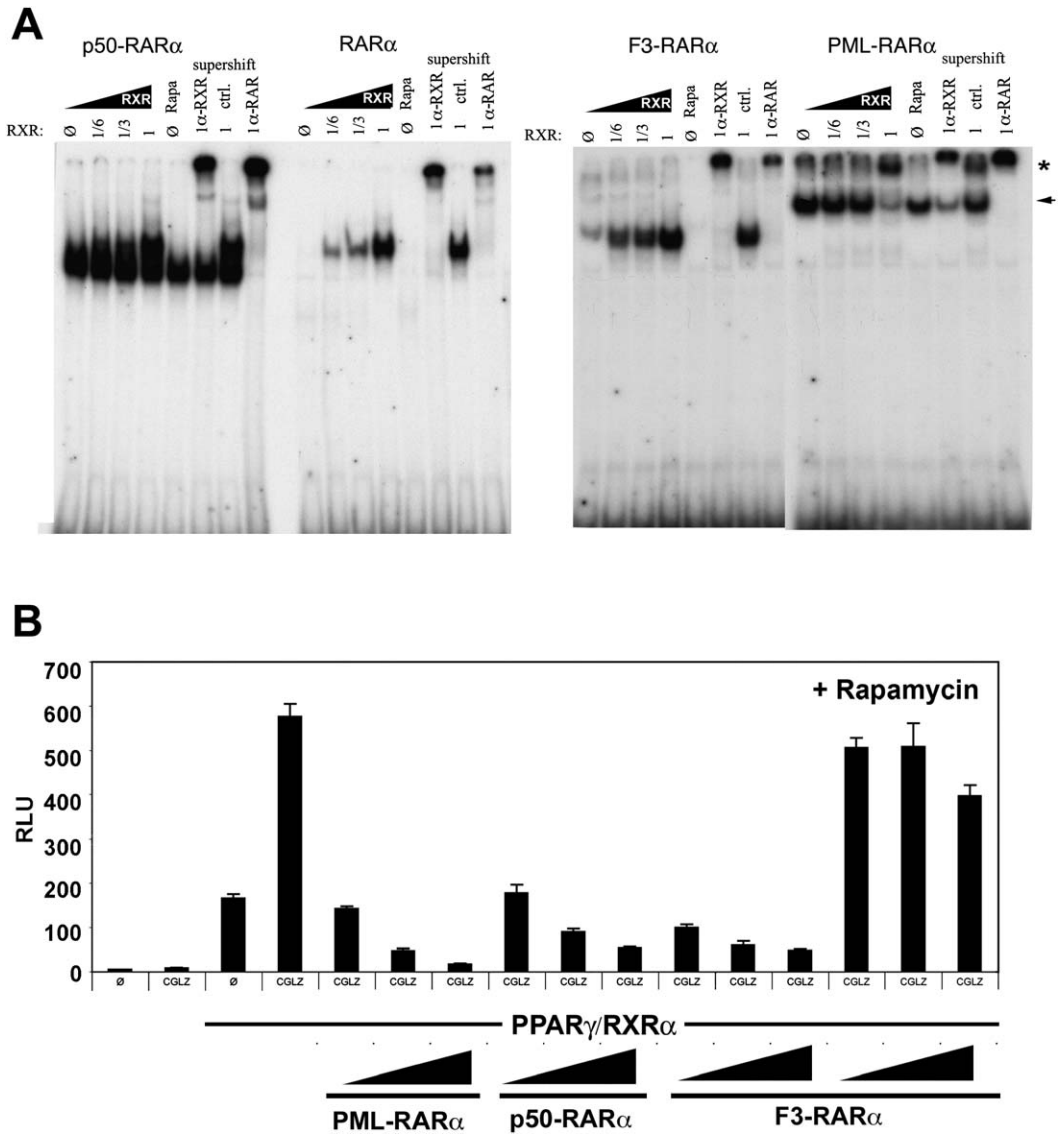

Figure 2. DNA binding and transcriptional properties of X-RARα fusion proteins

A: DNA binding of the X-RARα fusion proteins. Proteins were generated by in vitro translation. Increasing amounts of in vitro translated RXR, diluted in empty reticulocyte lysate, were added in a total volume of 1 μl prior to the addition of labeled oligonucleotide. DNA binding of F3-RARα, but not of PML-RARα or p50-RARα, is abrogated by addition of 2 μM rapamycin. A DR5 RARE was used for gel shift analysis. PML-RARα binds as a homodimer (arrow) and as an oligomer (asterisk). RARα does not bind DNA in the absence of RXR (Ø, panel RARα). Addition of polyclonal antibodies against RXR (αRXR) or RAR (αRAR) to the binding reaction results in a supershift of the corresponding complexes to the top of the lane, confirming that all analyzed fusion proteins are capable to form higher-order complexes with RXR.

B: X-RARα fusion proteins mimic transcriptional properties of PML-RARα. HEK293 cells were transiently transfected with a DR1(3x)-Tk-Luciferase reporter construct and expression constructs for the indicated proteins. The amount of each plasmid per well/48-well plate was as follows: PPARγ/RXRα, 10 ng each; PML-RARα, p50-RARα, or F3-RARα, each at 3, 10, or 30 ng. Repression of PPARγ by PML-RARα was described previously. PPARγ transactivation was induced by addition of the ligand Ciglitizone (CGLZ). PML-RARα, p50-RARα, and F3-RARα repress ligand-dependent transactivation by PPARγ in a dose-dependant manner. Repression by F3-RARα is completely abrogated by addition of 2 μM rapamycin, arguing that RARα homomerization through the FKBP12 domain is required for its repressive activity. The assay was performed in triplicate. Data are shown as mean ± standard deviation.

corresponding cells in the blue channel stained by Hoechst 33258 to visualize nuclei. Therefore, our immunostaining data suggest that neither p50-RARα nor F3-RARα directly affects endogenous PML.

Artificial X-RARα fusion proteins bind DNA as complexes with RXR and repress transcription

We assessed whether our artificial fusion proteins are capable of recapitulating crucial functional aspects of PML-RARα in vitro. The ability of the fusion proteins to bind to retinoic acid response elements (RAREs) was analyzed using electrophoretic mobility shift assays (EMSA). Given the finding that higher-order PML-RAR/RXR complexes contribute to the relaxed binding specificity of PML-RAR, we examined DNA binding of RARα, p50-RARα, F3-RARα, and PML-RARα to a DR5 element in vitro with or without RXR (Figure 2A; each protein is shown in a separate panel). Lane 1 for each protein shows binding in the absence of RXR, whereas lanes 2–4 in each panel show binding

in the presence of increasing amounts of RXR. Lane 5 shows the effect of rapamycin on binding when RXR is absent (compare to lane 1). Lanes 6-8 demonstrate the ability of antibodies to RXR (lane 6) or RAR (lane 8) to supershift the complexes as compared to control serum (lane 7).

A number of observations were evident. First, the three fusion proteins were capable of binding DNA in the absence of RXR. Second, the addition of RXR altered the intensity and/or migration of the complexes for all three fusions. Third, in all cases antibodies to RXR or RAR led to a specific supershift of the complexes toward the top of the lanes. Fourth, disruption of dimerization by addition of rapamycin eliminated binding of F3-RARα to the response element when RXR was absent, whereas it did not affect p50-RARα or PML-RARα binding. Fifth, the results for PML-RARα differ somewhat from those for p50-RARα and F3-RARα in that the majority of the complex migrates extremely slowly (almost as slowly as the supershifted complex) after addition of the highest amount of RXR. These results confirm that our artificial X-RARα fusion proteins bind to RAREs, demonstrate that the binding of F3-RARα can be regulated by rapamycin, and reveal similarities and differences in the complexes formed by the artificial fusions as compared to PML-RARα.

PML-RARα is known to repress transcription induced by a number of nuclear receptors, including RARs, VDR, and PPARs, on an array of nuclear receptor response elements. The ability of PML-RARα to influence transcription from more genes than those impacted by RARα is due in part to a tolerance for various spacing of nuclear receptor binding half-site motifs (Jansen et al., 1995; Kamashev et al., 2004; Lin and Evans, 2000). This relaxed binding site specificity may be a crucial aspect for leukemogenesis by X-RARα fusion proteins. To address if our X-RARα fusion proteins can similarly influence gene expression, we performed reporter gene assays using various nuclear receptor responsive reporter constructs. As depicted in Figure 2B, like PML-RARα, the artificial fusion proteins efficiently repress PPAR-dependent transcriptional transactivation from a DR1-containing promoter in a luciferase-based reporter gene assay (shown for PPAR-mediated transactivation). Similar repression was observed for RXR/RXR (DR1)-, RXR/LXR (DR4)-, and RXR/RAR (DR5)-dependent transactivation (data not shown). When 2 μM rapamycin was added, repression by F3-RARα was completely reversed (Figure 2B, last three columns), whereas rapamycin had no effect on repression by PML-RARα or p50-RARα (data not shown).

A myeloid cell line is immortalized by X-RARα fusion proteins

Although it has been reported that PML-RARα can immortalize normal myeloid progenitors (Altabef et al., 1996; Du et al., 1999; Kamashev et al., 2004), this approach, with various variations, was unproductive in our hands. As an alternative, we chose the ECoM-G cell line (Sykes and Kamps, 2001), which was established from BALB/C lineage(−) murine bone marrow cells, expressing an estrogen-responsive version of the pre-B cell lymphoma fusion oncoprotein E2a-Pbx1. In the presence of estrogen, ECoM-G cells are immortalized, whereas removal of estrogen leads to retention of E2a-Pbx1 in the cytoplasm, progressively followed by differentiation, growth arrest, and cell death.

ECoM-G cells were infected with MSCV-based retroviral vectors expressing PML-RARα, p50-RARα, or F3-RARα under control of the retroviral LTR. Infection was monitored by coexpression of the green fluorescent protein (GFP) from the same mRNA via an internal ribosomal entry site (IRES). Twenty-four hours after infection, cells were washed and reseeded in medium with or without estrogen. Strikingly, in the absence of estrogen, a GFP-positive population of dividing cells took over within 3 weeks (F3-RARα and p50-RARα) or 6 weeks (PML-RARα) (representative data shown in Figure 3A). No additional antibiotic selection was performed, indicating that in the absence of estrogen (after inactivation of the estrogen-responsive E2a-Pbx1) PML-RARα, p50-RARα, and F3-RARα provide a selective advantage to the expressing (GFP-positive) cells and permit immortalization.

Immature myeloid phenotype depends on homodimerization

To address if forced dimerization is responsible for the ability of X-RARα fusions to limit differentiation, we utilized the F3-RARα-transformed ECoM-G cells. These cells were kept under normal growth conditions or grown in the presence of 2 μM rapamycin. In the presence of rapamycin (which leads to disruption of dimerization), expression of the myeloid differentiation antigens CD11b (Figure 3B) or Gr1 (data not shown) is upregulated in ECoM-G/F3-RARα cells. In contrast, rapamycin has no differentiative effect on ECoM-G cells that express PML-RARα. This result indicates that dedimerization reverses the ability of X-RARα fusion proteins to impair differentiation.

p50-RARα and F3-RARα fusion proteins are poor initiators of myeloid leukemia

As evident from our in vitro studies, artificial X-RARα fusion proteins mimic many aspects of the PML-RARα protein, with the notable exception that they do not interfere with PML and its corresponding nuclear domains. To determine whether expression of X-RARα proteins can induce leukemia, transgenic mice were generated that express p50-RARα or F3-RARα under control of the MRP8 promoter (previously used to generate *PML-RARA* mice). Figure 4A shows Western blots demonstrating expression of the corresponding transgenes in the bone marrow of transgenic mice. Of note, two of the three lines of *F3-RARA* mice show only weak bands at the position of F3-RARα.

One hundred eighteen mice from three *p50-RARA* lines were followed for 225–837 days. The characteristics of this cohort are shown in Table 1. In transgenic line 1182, four animals developed myeloid leukemic pathology (at days 225, 394, 519, and 588), as assessed by cytology, histopathology, and flow immunophenotyping (Figure 4B and data not shown). In two of the mice for which peripheral blood was obtained, anemia and thrombocytopenia were observed. One of the animals had leukocytosis with significant numbers of maturing neutrophils. In the other animal, the white blood cell (WBC) count was not elevated, but circulating immature forms/blasts exhibited primary granules in their cytoplasm (similar to *MRP8 PML-RARA* leukemias), indicative of myeloid leukemia without maturation (Figure 4B, mouse #2641). The pathological features in the other three mice were most consistent with a diagnosis of myeloid leukemia with maturation (Figure 4B, mouse #1750), although myeloproliferative disease can not be excluded (Kogan et al., 2002). We assessed the ability of one immature leukemia (#2641) and one maturing leukemia (#2430) to engraft into sublethally irradiated recipients. One million splenic cells from the primary animals were injected into the lateral tail veins of

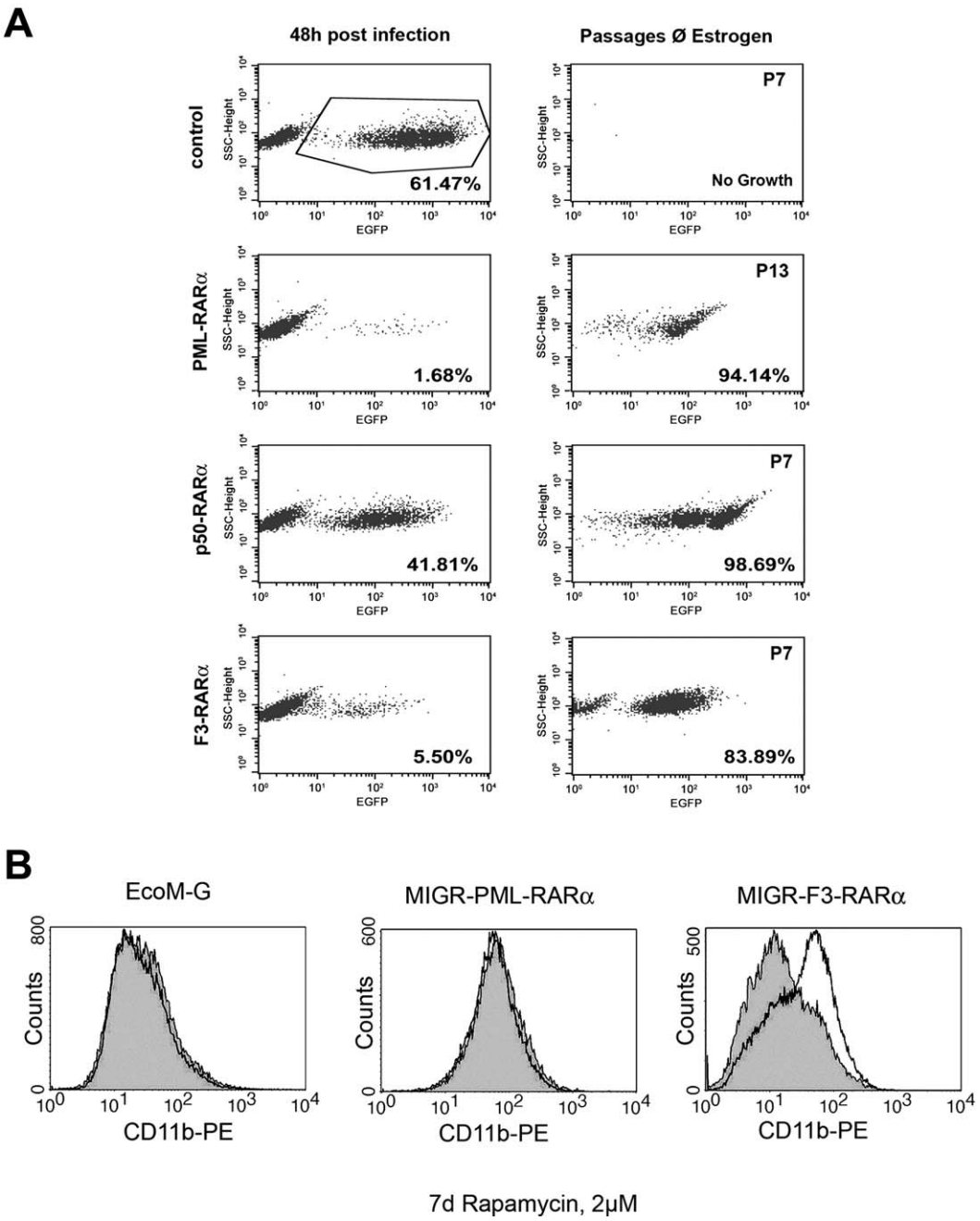

Figure 3. Effect on ECoM-G cells of X-RARα fusion proteins

A: Expression of F3-RARα and p50-RARα, like that of PML-RARα, immortalizes myeloid-committed ECoM-G cells. ECoM-G cells were infected with GFP expressing retrovirus MIGR-IRES-GFP, or the corresponding virus expressing PML-RARα, p50-RARα, or F3-RARα. After infection, differentiation was induced by removal of estrogen, and the cells were cultured for the indicated number of passages without further selective pressure. Plots were gated on live cells only (7AAD negative). Percentage of GFP-positive, 7AAD-negative cells (R2) is given in each dot plot. Cells were analyzed at passage numbers 1 and 7 postinfection for p50-RARα and F3-RARα and at passage numbers 1 and 13 postinfection for PML-RARα. Cells infected with virus expressing only GFP usually undergo growth arrest and die before passage 7. Cells infected with virus expressing p50-RARα, F3-RARα, or PML-RARα take over the population before P7 or P13, respectively.
B: F3-RARα-immortalized cells respond to rapamycin treatment. PML-RARα, p50-RARα, and F3-RARα cells were seeded in the respective growth medium in the presence or absence of rapamycin and cultivated for 7 days. For the histogram analysis, live cells were gated as determined by negative 7AAD staining. Only F3-RARα cells respond to rapamycin by increased expression of the differentiation marker CD11b.

sublethally irradiated (4.5 Gy) recipient animals. Three of five recipients of cells from #2641 or #2430 mice were observed to develop leukemia (first illness at 66 days for recipients of leukemia #2641, first illness at 126 days for recipients of leukemia #2430).

We also followed 115 mice from two *F3-RARA* lines (Table 1) as well as eight additional founder *F3-RARA* individuals for 113–548 days. Similar to three of the diseased *p50-RARA* mice, two *F3-RARA* mice (at days 229 and 717) developed disease consistent with a diagnosis of myeloid leukemia with maturation

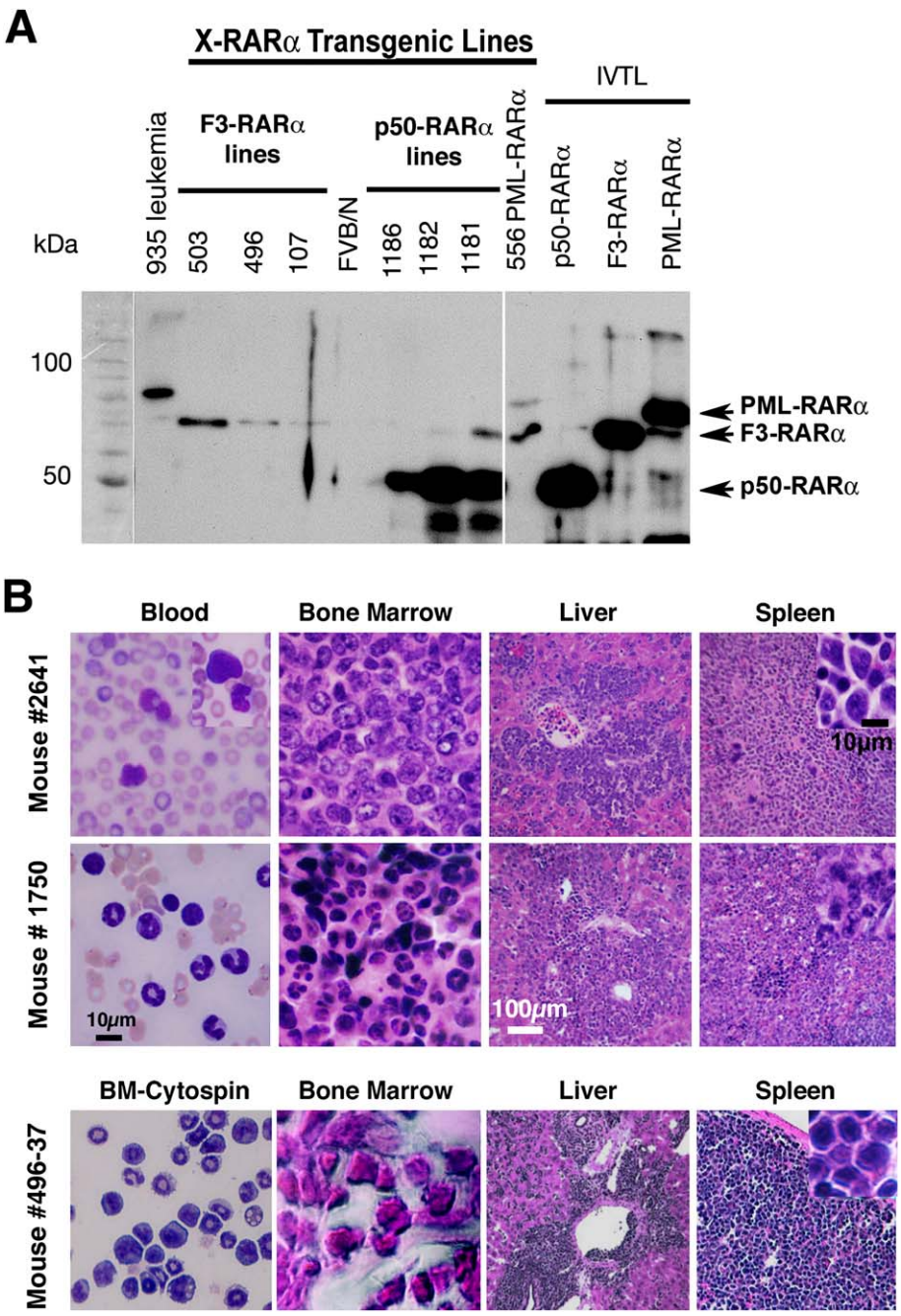

Figure 4. *p50-RARA* and *F3-RARA* transgenic mice

A: Expression of X-RARα fusion proteins in bone marrow of transgenic mice. Immunoblot of protein extracts. Extracts were derived from young healthy animals or leukemic mice, as noted: PML-RARα leukemia 935 of the 556 line; healthy F3-RARα transgenic mice of lines 503, 496, and 107; FVB/n control; healthy p50-RARα transgenic mice of lines 1186, 1182, and 1181; healthy PML-RARα transgenic mouse of line 556; or as a control, extracts from in vitro translated proteins (''IVTL'' lanes) using the Quick Coupled in vitro translation system were used diluted 1:200 in sample buffer, boiled, and loaded on the gel (three lanes on the right). Positions of the PML-RARα, F3-RARα, and p50-RARα proteins are marked by arrows. (Bands at a molecular weight similar to F3-RARα seen in lanes 1182 and 1181 represent a protein crossreacting with the polyclonal rabbit serum. Band at this position in the PML-RARα 556 sample may represent crossreacting material and a degradation product of PML-RARα.) Figure S1 is a second Western blot included to show expression of p50-RARα in a leukemic 1182 mouse, #2641.

B: Phenotype of leukemias in the p50-RARα and F3-RARα mice. Shown are blood smears or bone marrow cytospins (for mouse #496-37) stained with Wright's Giemsa, as well as hematoxylin and eosin-stained sections from sternum (bone marrow), liver, and spleen, as indicated. Mouse #1750 developed a differentiated-type myeloid leukemia, characterized by mature neutrophil-like cells in marrow and blood, similar to mouse #496-37, whereas mouse #2641 presented with myeloid leukemia of blastic-type. In all cases, infiltration of liver and spleen was observed.

(Figure 4B). Taken together, our artificial X-RARα constructs are leukemogenic, but compared to a *PML-RARA* transgene they give rise to leukemias at a reduced frequency and are less likely to give rise to disease characterized by arrested maturation.

Fusion of PML to RARα is a gain-of-function mutation

It has been suggested that the oncogenic activity of PML-RARα rests in its ability to act as a double dominant-negative protein, inhibiting the normal activity of both RARα and PML. We have shown that p50-RARα can inhibit RARα function but, lacking PML sequences, should not interfere with endogenous murine PML. In order to assess whether loss of PML function is what distinguishes p50-RARα and PML-RARα, we crossed line *p50-RARA* 1182, where we observed leukemias, into a *Pml* null background. PML has been reported to have a central role in formation of PML bodies (Ishov et al., 1999; Zhong et al., 2000a), and loss of PML leads to an early onset of disease in a PML-RARα transgenic/PML knockout model (Rego et al., 2001). Hence, we hypothesized that lack of PML might mimic the disruption of PML bodies by the oncoprotein and restore leukemogenesis. The *p50-RARA/Pml*$^{-/-}$ cohort of mice (Table 1, top, last column) showed no evidence of increased incidence of leukemia. We conclude from this that loss of PML does not complement p50-RARα in restoring leukemia-promoting activity to a level comparable to PML-RARα. The lack of leukemias in these *p50-RARA/Pml*$^{-/-}$ mice suggests that the PML-RARα fusion does not simply block PML and RARα function, but rather that the fusion protein represents a gain-of-function mutation.

Table 1. Survival of *X-RARA* transgenic mice

Survival of *PML-RARA* transgenic mice[a]				
Transgenic line	556			
Number of mice	36			
Deaths	36			
Leukemia	24			
Not leukemia	12			
Survival of *p50-RARA* transgenic mice				
Transgenic line	1181	1182	1186	1182/*Pml*$^{-/-}$
Number of mice	35	59	27	43
Deaths	27	50	17	8
Leukemia	0	4	0	0
Not leukemia	25	38	15	7
Unknown[b]	2	8	2	1
Follow-up: median, range (days)	554, 217–743	557, 225–761	497, 292–872	418, 290–659
Mice > 266 days[c]	34	58	25	43
Survival of *F3-RARA* transgenic mice				
Transgenic line	107	496	503	
Number of mice	35	63	17	
Deaths	9	9	2	
Leukemia	1	1	0	
Not leukemia	6	2	1	
Unknown[b]	2	5	1	
Follow-up				
Follow-up: median, range (days)	531, 370–878	391, 361–618	390, 370–544	
Mice > 266 days[c]	35	63	17	

[a]Leukemias developed in five of six lines of MRP8 *PML-RARA* transgenic mice (Brown et al., 1997). Line 556, which showed both the highest incidence of leukemia and the greatest level of transgene expression as assessed by Western blotting, was studied further. In order to avoid the limiting effect of skin hyperplasia, bone marrow from young line 556 mice was transplanted into lethally irradiated recipient animals, and recipients were followed for the development of leukemia. The results of these transplant studies are shown. The median time to leukemia in these transplanted animals was 266 days, with a range of 150–478 days.
[b]Mice for which leukemia as a cause of death could be neither documented nor disproven.
[c]Median time to leukemia in *PML-RARA* line 556 mice.

We note that *MRP8 PML-RARA* mice, due to activity of this promoter in skin, develop squamous papillomatous lesions (Hansen et al., 2003). These lesions can be of such severity as to interfere with breeding and to limit life span. We have not observed this skin phenotype of *p50-RARA* or *F3-RARA* mice in *Pml*$^{+/+}$ mice. In support of a gain-of-function model, *p50-RARA/Pml*$^{-/-}$ mice do not exhibit abnormal skin.

p50-RARα, like PML-RARα, cooperates with an activated cytokine receptor to generate leukemias with features of APL

We and others have previously observed that activated cytokine receptors cooperate with PML-RARα to rapidly induce myeloid leukemias in mice (Kelly et al., 2002; Phan et al., 2003; Sohal et al., 2003). We therefore wanted to address if p50-RARα, although a poor inducer of leukemia on its own, might exhibit cooperative leukemogenesis with cytokine receptor activation. To assess this hypothesis, we transduced *p50-RARA* transgenic bone marrow with a retrovirus carrying an activated allele of the β_c chain of the GM-CSF/IL-3/IL-5 receptor (β_cV449E, CDw131) (Figure 5A). In these experiments, p50-RARα was observed to readily cooperate with activated β_c to cause leukemia. The combination of activated β_c with the *p50-RARA* 1182 transgene caused leukemia with longer latency than β_cV449E plus the *PML-RARA* 556 transgene (median 85 days versus median 47 days, $p < 0.0001$). However, it is not clear that this difference was due to differences in their protein structure (see below).

The leukemias that arose in recipients of *p50-RARA* bone marrow transduced with β_cV449E appeared similar to those in PML-RARα/β_cV449E recipients. The peripheral blood displayed leukocytosis including immature forms/blasts, anemia, and thrombocytopenia (Table 2). Bone marrows of p50-RARα/β_cV449E leukemias contained large immature forms/blasts with prominent primary granules (Figure 5B). These leukemias filled the red pulp of the spleen, engorged the liver (see Figure 5E, below), and were invasive in other tissues including lymph nodes and kidneys. The leukemias arising in p50-RARα/activated β_c mice were readily transplantable and, like PML-RARα leukemias, were responsive to retinoic acid. All-*trans*-retinoic acid (ATRA) treatment of leukemias resulted in differentiation and regression (Figures 5C–5H; Table 2) and enhanced survival time (median survival of placebo-treated mice was 35 days versus 47 days for ATRA-treated animals; $p = 0.04$).

In our transduction experiments using transgenic bone marrow, expression of p50-RARα is directed to the myeloid lineage by the hMRP8d promoter. Therefore, the development of myeloid leukemias might merely reflect restricted promoter activity. PML-RARα, on the other hand, was reported to dictate myeloid phenotype (Grignani et al., 2000; Minucci et al., 2002). In order to further establish that p50-RARα could cooperate with an activated cytokine receptor to cause myeloid leukemia, we generated retroviral vectors that expressed p50-RARα or the bcr3/short isoform of PML-RARα. Introduction of bone marrow that had been doubly transduced with p50-RARα/β_cV449E or PML-RARα/β_cV449E into mice led to leukemias within 9 weeks postinfection/posttransplantation. Transduction leads to variable levels of gene expression (dependent upon site of proviral integration), and in contrast to the different latencies observed

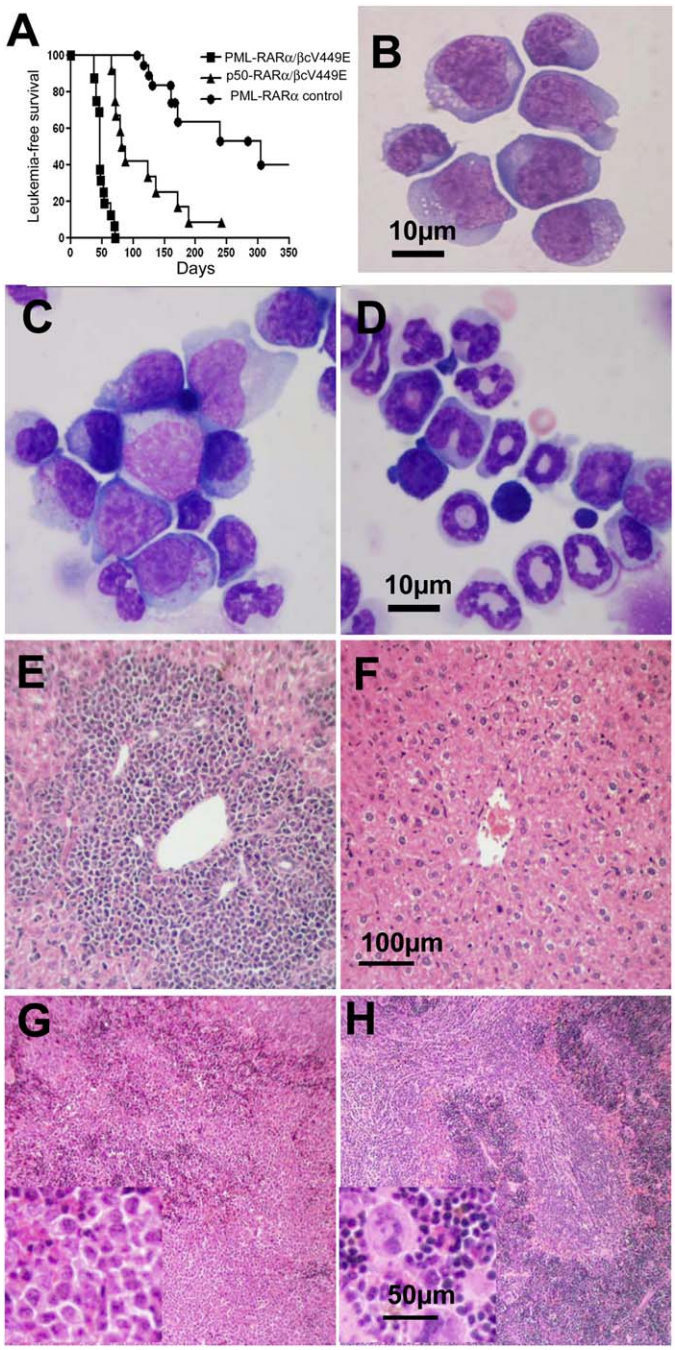

Figure 5. p50-RARα cooperates with an activated receptor to cause leukemia

A: p50-RARα cooperates with a transduced retrovirus encoding an activated cytokine receptor to cause leukemia. Bone marrow of *PML-RARA* transgenic (line 556) or *p50-RARA* transgenic (line 1182) mice were transduced with retroviruses expressing β_cV449E, and recipient mice were followed for the development of leukemia. For comparison, results in recipients of marrow from *PML-RARA* transgenic (line 556) animals transduced with control retroviruses (pRUFneo-$\beta_c$wt or MSCV-IRES-GFP) are shown. Nonleukemic deaths were censored at the time of death. Graphs shown include PML-RARα/β_cV449E (n = 16), p50-RARα/β_cV449E (n = 12), and PML-RARα/control (n = 19). Results with β_cV449E and control-transduced *PML-RARA* bone marrow include some previously published animals (8 of 16 animals for PML-RARα/β_cV449E, 4 of 12 animals for PML-RARα/control; Phan et al., 2003). The median latency of the 19 leukemias in recipients of PML-RARα marrow transduced with control retroviruses was 304 days, which is not significantly different from the latency of leukemias in untransduced PML-RARα marrow (266 days). Hence, although the mutagenic effect of retroviral transduction may be of relevance, under the conditions utilized for our studies it is expected that the activated nature of the β_cV449E allele plays an important role in generating leukemias from marrow expressing the *p50-RARA* transgene; it is unlikely that the high penetrance and short latency of the p50-RARα/β_cV449E leukemias results solely from insertional mutagenesis.

B: p50-RARα cooperates with an activated cytokine receptor to generate immature forms/blasts with features of promyelocytes. A cytospin was prepared with bone marrow cells of a leukemic p50-RARα/β_cV449E mouse, and cells were stained with Wright's Giemsa stain.

C–H: p50-RARα/activated cytokine receptor leukemias respond to retinoic acid. Mice were injected with p50-RARα/β_cV449E leukemic cells. After 21 days, the mice were treated with placebo (**C**, **E**, and **G**) or ATRA (**D**, **F**, and **H**). Four days later, the mice were euthanized. Representative images are shown. **C** and **D:** Bone marrow cytology, Wright's Giemsa stain. Differentiating neutrophils are evident in the ATRA-treated animal. **E** and **F:** Liver histopathology. ATRA markedly reduces leukemic infiltrate. Hematoxylin and eosin. **G** and **H:** Splenic histopathology. ATRA restores nearly normal architecture in the spleen. Hematoxylin and eosin.

above when transgenic marrows were utilized, the latencies of leukemias when normal bone marrows were doubly transduced with p50-RARα/β_cV449E or PML-RARα/β_cV449E were not significantly different (n = 9 for both groups; median 53 days versus median 69 days; p = 0.20). This finding suggests that different levels of expression of X-RARα fusions (e.g., as transgenes versus as retroviral vectors) influences their oncogenic potency.

The p50-RARα/β_cV449E and PML-RARα/β_cV449E double virus-induced leukemias exhibited overall similar cytology and histopathology (data not shown), although a subtle decrease in differentiation may be associated with the PML fusion relative to the p50 fusion. Flow cytometric immunophenotyping revealed diseases that exhibited essentially identical expression of cell surface markers (Table S1 in the Supplemental Data available with this article online). Our findings with doubly transduced bone marrow cells confirm that an artificially dimeric X-RARα fusion, like the PML-RARα fusion, can cooperate with an activated cytokine receptor to generate myeloid leukemias. Interestingly, Q-PCR analysis of p50-RARα/β_cV449E leukemias as compared to PML-RARα/β_cV449E leukemias showed nearly identical patterns of gene expression (Figure 6). Taken together, these data support the idea that, in the context of additional mutations, dimerization of RARα through its fusion partners both contributes to transformation and underlies the promyelocytic phenotype of APL.

Discussion

Since the discovery that, in addition to the classical PML-RARα fusion, other RARα fusions can be found in APL patients (reviewed in Melnick and Licht, 1999), the significance of RARα fusion partner pathways for promotion of APL development has been a matter of intense debate. These partners, lacking further obvious functional resemblance, all introduce a homooligomerization domain into the fusion protein. This raised questions about the degree of fusion partner contribution in general—and that of PML in particular—to leukemogenesis (Cheng et al., 1999; Grignani et al., 1996; Pandolfi, 1996; Piazza et al., 2001; Sachdev et al., 2001; Seeler and Dejean, 1999; Strudwick and Borden, 2002; Wang et al., 1998; Zhong et al., 2000b). Specifically, the role of the disruption of PML bodies/PODs by PML-RARα has been debated since PML protein localization was first

Table 2. Peripheral blood counts and bone marrow differential counts in p50-RARα/β_cV449E mice

Leukemias in PML-RARα/β_cV449E and p50-RARα/β_cV449E mice are characterized by leukocytosis, anemia, and thrombocytopenia					
	Normal	PML-RARα/β_cV449E	p50-RARα/β_cV449E		
WBC ($\times 10^3/\mu l$)	3.9 ± 2.5	86.0 ± 45.0	51.6 ± 8.9		
HGB (mg/dl)	13.5 ± 0.3	9.0 ± 2.4	5.9 ± 2.3		
PLT ($\times 10^3/\mu l$)	1041 ± 83	237 ± 83	206 ± 56		
ATRA causes differentiation and regression of p50-RARα/β_cV449E leukemias					
	Immature forms/blasts[a]	Intermediate forms[a]	Mature neutrophilic[a]	Spleen weight[b]	Liver weight[b]
Placebo	82.2 ± 7.5	7.8 ± 0.8	9.4 ± 7.3	0.67 ± 0.12	1.4 ±0.1
ATRA	20.2 ± 4.7[c]	12.2 ± 3.4	55.8 ± 4.9[c]	0.20 ± 0.06[c]	1.2 ± 0.1[c]

Mean ± SD.
Normal, n = 9; PML-RARα/β_cV449E leukemias, n = 12; p50-RARα/β_cV449E leukemias, n = 4; placebo and ATRA, n = 5.
Normal and PML-RARα/β_cV449E leukemia data have been previously published (Phan et al., 2003).
[a]Percentage of total nucleated marrow cells.
[b]Grams.
[c]$p < 0.05$ compared to placebo.

described (Dyck et al., 1994; Koken et al., 1994). We have addressed this question by replacing the PML domain of PML-RARα with two biologically unrelated dimerization/oligomerization domains.

Our study comes to the conclusion that interaction with the cellular PML complex is not required for many PML-RARα activities, such as RXR-independent DNA binding, transcriptional repression of a range of nuclear receptor response elements, immortalization of ECoM-G cells, and inhibition of differentiation of myeloid cell lines. Our data imply that the involvement of the PML pathway in these in vitro activities is of lesser, if any, importance. In addition, by demonstrating that abrogation of F3-RARα dimerization with rapamycin impairs the in vitro activities of the fusion, our results underscore the important contribution of the dimerization interface to the abnormal properties of the chimeric proteins.

Interestingly, in vivo analyses of the leukemogenic potential of p50-RARα and F3-RARα fusion proteins indicate that dimerization of RARα is only one of the important properties of PML-RARα. Although p50-RARα clearly was able to contribute to leukemogenesis by sensitizing the cells to an activated cytokine receptor, it was a poor initiator of leukemia on its own. Likewise, the second fusion protein, F3-RARα, resulted in myeloid disease at very low frequency and was able to sensitize cells to an activated cytokine receptor (data not shown). Both fusion proteins, although biologically active in vitro, obviously lack an important feature for leukemic induction, which is encoded in the PML domain. Further evidence for the importance of the PML domain to the initiation of leukemia is provided by the lack of leukemias in transgenic animals expressing a homodimerizing HDAC-RARα fusion protein (Pier Paolo Pandolfi, personal communication). These observations suggest that PML may be the most common partner of RARα because the PML-RARα fusion appears to be the most potent initiator of leukemic transformation.

Several possibilities could explain the oncogenic potency of the PML fusion. First, the PML domain may play a central role in the initiation of leukemogenesis by virtue of disruption of the PML nuclear bodies. Several studies have pointed out that

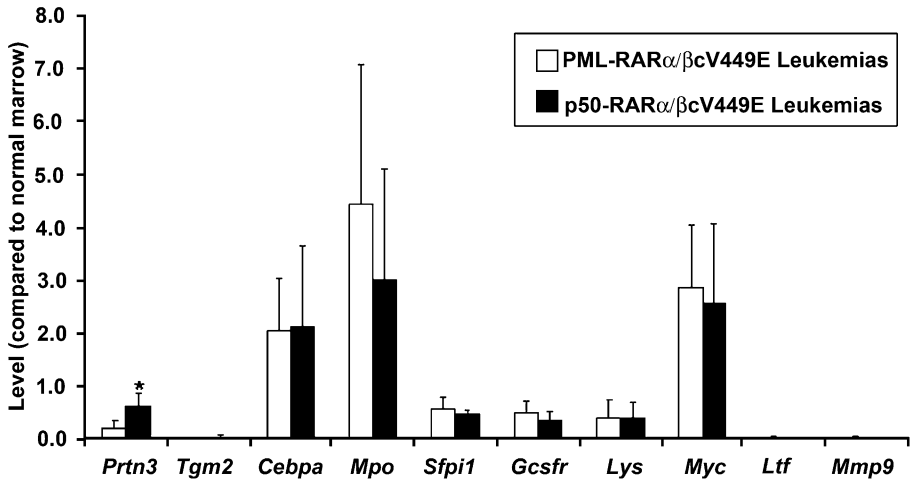

Figure 6. PML-RARα/β_cV449E and p50-RARα/β_cV449E leukemias express similar levels of myeloid genes

RNA was prepared from PML-RARα/β_cV449E leukemic bone marrow (three leukemias from *PML-RARA* transgenic marrow transduced with β_cV449E plus three leukemias from marrow doubly transduced with PML-RARα + β_cV449E) or p50-RARα/β_cV449E leukemic bone marrow (three leukemias from *p50-RARA* transgenic marrow transduced with β_cV449E plus two leukemias from marrow doubly transduced with p50-RARα + β_cV449E). Results are presented in comparison to normal bone marrow. Genes associated with terminal neutrophil differentiation (*Tgm2*, *Ltf*, *Mmp9*) are markedly decreased in the leukemias compared to normal marrow. The expression of myeloblastin (*Prtn3*) is significantly lower in PML-RARα/β_cV449E leukemias than in p50-RARα/β_cV449E leukemias, suggesting that different X-RARα fusions can have differential effects on particular target genes ($p < 0.05$). Data are shown as mean ± standard deviation.

PML is involved in regulation of apoptosis (Guo et al., 2000; Quignon et al., 1998; Wang et al., 1998) and senescence (Ferbeyre et al., 2000). A disruption of these postulated PML activities could very well establish a premalignant state that facilitates the acquisition of or tolerance to additional genetic changes. Second, recruitment of PML-associated factors to the PML-RARα/RXR complex and its target genes may have an important impact on gene expression. Such PML-RARα-specific alterations in the transcription of genes that regulate apoptosis, proliferation, or differentiation may underlie the ability of PML-RARα to initiate leukemia. In consonance with this idea is recent work indicating that mutation of K160 of PML, the major site of sumolation, disrupts the ability of PML-RARα to enhance proliferative potential in vitro and to initiate acute myeloid leukemias in vivo (Zhu et al., 2005). Another possibility is that the PML domain confers a specific conformational architecture to RARα DNA binding complexes, for instance by the formation of higher-order multimers as proposed previously (Minucci et al., 2000). With regard to this possibility, our gel shift experiments show that the RXR/p50-RARα and RXR/F3-RARα protein complexes are not identical to those formed by RXR/PML-RARα (Figure 3A). Hence, an important conformational effect of the PML domain cannot be ruled out. Taken together, the data obtained in this study indicate a very significant role of the PML domain in leukemogenesis, perhaps through more than one molecular mechanism.

PML-RARα was often suggested to be a double dominant-negative mutant for both the RARα and the PML pathways (Pandolfi, 2001). Lack of PML was reported to correspond to decreased levels of apoptosis in response to ionizing radiation and Fas/TNF treatment (Wang et al., 1998). In addition, lack of PML seems to prevent the localization of other PML-body associated proteins to dot-like domains, instead leading to a diffuse distribution (Ishov et al., 1999; Zhong et al., 2000a). To further address the significance of the PML pathway in leukemogenesis, we bred the *p50-RARA* allele from the 1182 transgenic line (the line in which we observed spontaneous leukemias) into the *Pml* null background. We did not observe increased leukemias in the *p50-RARA*/*Pml* null cohort. To avoid the complexities associated with strain variability, we backcrossed the *Pml* null allele more than ten generations into the FVB/N background to obtain the line with which we performed these experiments. These data argue against inactivation of PML being a sufficient factor. Of note, because gene inactivation of *Pml* is not necessarily equivalent to active disruption of PML nuclear bodies, our data do not exclude the possibility that nuclear body disruption has a role in pathogenesis. In the absence of a robust functional assay for PML (or the nuclear bodies of which they are a part) to directly address this question, our data here provide compelling evidence that the activity of PML-RARα is not merely that of a double dominant-negative protein. Rather, our results indicate that PML-RARα represents a true gain-of-function mutation. The observation that low-level expression of PML-RARα induces leukemia in mice is consistent with this gain-of-function hypothesis (Westervelt et al., 2003).

Our findings support the hypothesis that dimerization of RARα is critical to the leukemic phenotype of APL. Dimerized RARα is tightly associated with the morphologic features of APL in humans and, in the right setting, with an APL-like disease in mice. Dimerization of RARα through its fusion partners results in alterations in binding site specificity (Hauksdottir and Privalsky, 2001; Jansen et al., 1995; Kamashev et al., 2004). This change in binding site specificity also represents a gain-of-function phenotype for X-RARα fusions because it extends the repressive ability of these chimeras beyond the target genes repressed by endogenous RARα. Future studies should elucidate how this expanded binding site specificity is important for X-RARα oncogenesis.

Elegant experiments in mice have demonstrated that the level of fusion protein expression (Westervelt et al., 2003) and proteolysis of PML-RARα by neutrophil elastase (Lane and Ley, 2003, 2005) make important contributions to the biological activities of PML-RARα, including its ability to cause leukemic transformation. There may in fact be an important balance of activity brought about by the combined impact of the level of protein production and rate of proteolysis. In our experiments in ECoM-G cells, we noted that initially, in contrast to findings with the weakly leukemogenic artificial RARα fusions, few PML-RARα-expressing cells were present 48 hr after transduction. This difference was due to induction of apoptosis by PML-RARα in these cells (data not shown), and therefore more passages were required to observe domination of PML-RARα cells in the cultures than for either of the artificial X-RARα fusions. This observation is in line with previous studies indicating a toxic effect of PML-RARα on many cell types that is dependent upon its PML domain (Ferrucci et al., 1997). It is an intriguing possibility that the level of expression of PML-RARα is of critical importance in human APL, and that the activities responsible for the toxicity of the protein in certain settings might be the very same activities that make PML-RARα more leukemogenic than a random dimerizing X-RARα fusion. Ongoing (noted in Lane and Ley, 2005) and future work may further delineate such a connection.

Our data support the idea that PML-RARα-induced leukemogenesis is a multistep process, requiring cooperating events that can also influence the phenotype. The rare spontaneous leukemias that we observed in the p50-RARα transgenic cohort were either of the blastic type (1 of 4) or of a more differentiated type (3 of 4). This finding has some resemblance to the observations in the PLZF-RARα transgenic animals (Cheng et al., 1999; He et al., 1998). In transgenic animals, the expression of PLZF-RARα alone leads to development of a leukemia of a more differentiated type, whereas coexpression of the reciprocal fusion product RARα-PLZF produces a phenotype closely resembling APL (He et al., 2000). In the context of a very strong cooperating event such as activated β_c, a dimeric RARα contributes to disease that is remarkably similar to that created with PML-RARα under the same conditions, a disease with features of human APL including responsiveness to ATRA therapy.

We propose that the PML-RARα oncoprotein has complex activity that extends beyond disruption of PML and RARα functions. These activities are in part described by our in vitro assays but also involve an additional activity (or activities) of the PML part of the fusion. Our results highlight the fact that a single genetic event may contribute to transformation through multiple mechanisms. We speculate that karyotypically simple AMLs contain a few genetic lesions, each of which has multiple proleukemic effects, whereas the karyotypically complex AMLs may contain a large number of genetic lesions, each of which has a simpler contribution to transformation.

It is evident that forced dimerization by fusion partners represents a tempting target for pharmacological intervention. Our

in vitro experiments show that disruption of F3-RARα dimerization by rapamycin is sufficient to inactivate RXR-independent DNA binding, repression, and block of myeloid differentiation. A screen for chemicals that selectively bind the dimerization domain of PML and therefore selectively inhibit pathological RARα dimerization could be a promising strategy for a targeted therapy of PML-RARα-induced APL. More generally, pharmacologic disruption of critical protein-protein interactions, including abnormal dimerization, may enhance treatment response in AML and other malignancies.

Experimental procedures

Recombinant plasmids

The p50-RARα construct has been described previously (Lin and Evans, 2000). Plasmid pSH-SF1E encoding the cDNA for FKBP12 followed by a hemagglutinin tag was a gift from Dr. Steffan Ho, UCSD, Department of Pathology. The F36M mutation was introduced using the QuikChange protocol (Stratagene, La Jolla, CA). Trimerization was achieved using the 5′-Xho1 and 3′-Sal1 restriction sites. The resulting trimer was placed into the plasmid pCMX-p50-RARα, replacing the p50 part, which gave rise to plasmid pCMX-F3RAR. The translated breakpoint including the HA tag corresponds to the amino acid sequence (FKBP12=)VELLKLEVDYPYDVPDYALDEFIET QSSS(=RARα). This plasmid was used as a template for transfer into pMIGR and phMRP8d by PCR cloning. pCMX-p50-RARα was modified by insertion of a double-stranded oligonucleotide at the 5′ end to permit transfer as a BglII-BamHI fragment into pMIGR and phMRP8d. Plasmids pMIGR-PML-RARα(s) and pMIGR-PML-RARα(l) were generated by insertion of an EcoR1 fragment encoding the short (bcr3) PML-RARα isoform or the long (bcr1) PML-RARα isoform into the corresponding restriction site of pMIGR, an MSCV-based retroviral vector. pMIGR expresses inserted genes from the viral LTR and contains an open reading frame for the enhanced green fluorescence protein (EGFP) downstream of the multiple cloning site and an IRES derived from EMCV. Infected cells can therefore be monitored by their GFP expression. The pRufNeo retroviral constructs expressing $\beta_c$wt or β_cV449E (CDw131) have been described before (Jenkins et al., 1995, 1999). Plasmids encoding PML-RARα, PPAR, LXR, RAR, RXR, and pPPRE(Aox3x)tk-Luc used in the reporter gene assay have been described previously (Lin and Evans, 2000; Lin et al., 1998; Nagy et al., 1997 and references cited therein). Detailed sequence information is available upon request.

Cell culture, retroviral transduction, and immortalization of ECoM-G cells

The estrogen-responsive ECoM-G cell line was kindly provided by David Sykes and Mark Kamps (Sykes and Kamps, 2001). ECoM-G cells were cultivated in IMDM (Gibco BRL) supplemented with 10% fetal bovine serum, 1% GM-CSF conditioned medium, and estrogen (1 μM). Retroviral packaging was performed by transient transfection of 293 or BOSC23 cells (Pear et al., 1993) using the pEcopac ecotropic packaging construct (Finer et al., 1994) and the calcium phosphate method. Retroviral infection was performed by spinoculation for 99 min at 2500 g in a total volume of 1–2 ml in the presence of 0.1% v/v Lipofectamine. After spinoculation, viral supernatant was aspirated, and the cells were resuspended in IMDM/GM-CSF/FBS without estrogen and split as needed. No antibiotic selection was performed, so that changes in percentage directly reflect growth advantage or disadvantage conferred by expression of the respective X-RARα protein. Expansion of infected cells was monitored by FACScan analysis of GFP^+ cells at every passage.

Gel shifts and reporter gene assays

Gel shifts and reporter gene assays were carried out essentially as described before (Lin et al., 1998; Nagy et al., 1997). Proteins were produced by in vitro translation using TNT Quick Coupled reticulocyte lysate (Promega, Madison, WI). Bound complexes were run in 0.5× TBE PAA gels. For supershift experiments, polyclonal antibodies against RARα and RXR were purchased from Santa Cruz (Santa Cruz, CA) and diluted 1:20 (1 μl antibody per 20 μl binding reaction). As a negative control, polyclonal antiserum against Pu.1 was used. For reporter gene assays, usually 10 ng of receptor plasmid and 50 ng of reporter plasmid were transfected per well in 48-well plates into HEK293 cells, using the TransIt LT1 transfection reagent (Mirus Corporation, Madison, WI). Total DNA per well was filled up to 300 ng with salmon sperm DNA. Transfection efficiency was normalized by cotransfection of 5 ng pCMX-βGal per well. All transfections were carried out in triplicate.

Mice

FVB/N mice were purchased from Jackson Laboratories or Harlan. Transgenic mice were generated by microinjection of linearized hMRP8d-p50-RAR or hMRP8d-F3-RAR expression cassette into the pronuclei of fertilized FVB/N oocytes, following standard procedures. All animal experiments and care were carried out as approved by the corresponding UCSF and Salk Institute Animal Protocol Review Committee and in accordance with AAALAC guidelines. hMRP8d-PML-RARα mice have been previously described (Brown et al., 1997). Mice were observed daily for signs of illness. When any abnormality was observed, mice were subjected to a brief physical examination. Blood was obtained on animals that showed signs of illness and also in selected animals to screen for unsuspected disease. Mice were sacrificed when moribund or when physical examination and blood cell counts indicated likely rapid progression of illness.

Western blot analysis

Bone marrow was obtained by flushing buffered saline through mouse long bones. Total cell lysates from equal numbers of unfractionated bone marrow cells from control and transgenic mice were used. Western blot analysis was performed as described (Robbins et al., 1995) with rabbit polyclonal antiserum raised against a glutathione S-transferase fusion protein encompassing amino acids 420–462 of the human RARα protein (Gaub et al., 1992). In vitro translated samples were diluted 1:200 in SDS sample buffer.

Transduction of primary bone marrow

Donor mice were treated with 150 mg of 5-fluorouracil/kg, and marrow was harvested 5 days later. Marrow was prestimulated for 24 hr in Myelocult 5300 or StemSpan (Stem Cell Technologies) with 100 U of penicillin G/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine, 6 ng of mIL-3/ml, 10 ng of mIL-6/ml, and 10 ng of stem cell factor/ml in 12- or 24-well plates at 1–2 × 10^6 cells/well. Fifteen percent heat-inactivated fetal bovine serum and 5% X63Ag8-mIL-3 conditioned medium was also present for most experiments. BOSC23 cells were transfected with retroviral constructs. Marrow was transduced by spinoculation with either 2 ml of fresh retroviral supernatants (filtered through 0.45 μm filters) or 1 ml of each retroviral supernatant at 1100 g in the presence of 2 μg of Polybrene/ml for 1.5 hr on 2 consecutive days. Infection efficiency was assessed by FACScan analysis 24 hr after the second spinoculation. Unsorted cells after transduction were introduced into lethally irradiated mice (total of 9 Gy, in two equal doses 3–6 hr apart).

Peripheral blood counts and bone marrow differential counts

Blood was obtained from the retro-orbital sinus and anticoagulated with EDTA or heparin. WBC count, hemoglobin level, and platelet counts were measured with the Hemavet 850 cell counter (CDC Technologies) or by Antech diagnostics. Blood smears and bone marrow smears or cytospins and were stained with Wright's Giemsa stain or DiffQuick staining (Dade Behring). Peripheral blood differential WBC counts and bone marrow differential counts were determined by morphology as previously described (Kogan et al., 2002).

Histopathology

Tissues were initially fixed in either 10% buffered formalin solution (Fisher Scientific), 4% paraformaldehyde in PBS, or Bouin's fixative. Sternums fixed in formalin were decalcified for 3 hr prior to embedding (11% formic acid, 8% formaldehyde). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E), following standard protocols.

Immunophenotyping

After depletion of red blood cells, 100,000 to 300,000 bone marrow and/or spleen cells were resuspended in 100–200 μl of fluorescence-activated cell-sorting buffer (buffered saline with 2% heat-inactivated fetal bovine serum and 2.5% cell dissociation buffer [Gibco-BRL]). Cells were incubated with unlabeled anti-CD16/CD32 antibodies (Fc block) for 15 min prior to

the addition of conjugated antibodies. The antibodies used included fluorescein isothiocyanate (FITC)-conjugated antibodies to CD24, Ly-71(F4/80), CD71, CD34, CD16/32, I-A, CD41, IgD, CD45R(B220), CD8a, CD90, IgK, and TCRα/β; phycoerythrin (PE)-conjugated antibodies to Ly-71(F4/80), Ly-76(Ter119), CD31, Ly6A/E (sca-1), CD117(c-kit), CD86, IgM, CD5, CD3, and CD138(syndecan-1); Tricolor-conjugated antibodies to Ly6G(Gr-1), CD11b(Mac-1), CD45, CD4, CD19, and TCR γ/δ; biotin-conjugated antibodies to Ly6G (Gr-1), CD59, and human βc. Antibodies were incubated with the cells for 20 min in the dark on ice. Stained cells were washed, and then only cells stained with biotin-conjugated antibodies were stained with Streptavidin-APC for another 20 min in the dark. The cells were analyzed on a FACSCalibur apparatus (Becton Dickinson), and at least 10,000 events were collected for each sample. Fluorescence-activated cell sorting data were analyzed with CellQuest (Becton Dickinson). Alternatively, approximately 300,000 cells were resuspended in PBS/2% FBS and stained with a 1:100 dilution of the corresponding phycoerythrin or FITC/Cy2-conjugated antibody (Pharmingen). For viability staining, cells were incubated in a solution of 7 aminoactinomycin D (7AAD) (20 μg/ml) in PBS/2% FBS. Analysis was performed using a BD-FACScan flow cytometer (BD Biosciences) and the CellQuest or FloJo software packages. FSC and SSC gates were set to exclude residual erythrocytes and dead cells where necessary.

Quantitative polymerase chain reaction

Bone marrow cells harvested from the long bones of normal or leukemic mice were initially cryopreserved. Subsequently, the cells were thawed and centrifuged (600 g), and RNA was immediately isolated by extraction with TriZol reagent (#TR118, Molecular Research Center Incorporated) according to the manufacturer's protocol (http://www.mrcgene.com/tri.htm). Reverse transcription (RT) and quantitative polymerase chain reaction (Q-PCR) were performed at the UCSF Comprehensive Cancer Center Genome Core Facility. TaqMan Gene Expression Assays from Applied Biosystems for the following genes were utilized: *Cebpa*, *Gcsfr*, *Ltf*, *Lys*, *Mmp9* (Gelatinase B), *Mpo*, *Myc*, *Prtn3* (Myeloblastin), Sfpi1 (PU.1), and *Tgm2*. Percent of gene expression was normalized to mouse *Gapdh* expression. Each gene was assessed in triplicate for each sample.

Pathologic diagnosis

Gross pathology, blood cell counts, blood smears, bone marrow and splenic cytology, histopathology, and flow cytometric immunophenotyping were used for diagnosis of hematopoietic neoplasms according to published guidelines (Kogan et al., 2002).

Briefly, myeloid leukemia was diagnosed by hepatosplenomegaly with infiltration of leukemic cells into peripheral tissues outside the spleen and lymph nodes, as well as expansion of leukemic cells in the bone marrow with reduction of normal hematopoietic elements (resulting in peripheral anemia and thrombocytopenia).

Supplemental data

The Supplemental Data include one supplemental figure and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/2/81/DC1/.

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