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Opposite Effects of the Acute Promyelocytic Leukemia PML-Retinoic Acid Receptor α (RARα) and PLZF-RARα Fusion Proteins on Retinoic Acid Signalling

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Fusion proteins involving the retinoic acid receptor α (RARα) and the PML or PLZF nuclear protein are the genetic markers of acute promyelocytic leukemias (APLs). APLs with the PML-RARα or the PLZF-RARα fusion protein are phenotypically indistinguishable except that they differ in their sensitivity to retinoic acid (RA)-induced differentiation: PML-RARα blasts are sensitive to RA and patients enter disease remission after RA treatment, while patients with PLZF-RARα do not. We here report that (i) like PML-RARα expression, PLZF-RARα expression blocks terminal differentiation of hematopoietic precursor cell lines (U937 and HL-60) in response to different stimuli (vitamin D_3, transforming growth factor β1, and dimethyl sulfoxide); (ii) PML-RARα, but not PLZF-RARα, increases RA sensitivity of hematopoietic precursor cells and restores RA sensitivity of RA-resistant hematopoietic cells; (iii) PML-RARα and PLZF-RARα have similar RA binding affinities; and (iv) PML-RARα enhances the RA response of RA target genes (those for RARβ, RARγ, and transglutaminase type II [TGase]) in vivo, while PLZF-RARα expression has either no effect (RARβ) or an inhibitory activity (RARγ and type II TGase). These data demonstrate that PML-RARα and PLZF-RARα have similar (inhibitory) effects on RA-independent differentiation and opposite (stimulatory or inhibitory) effects on RA-dependent differentiation and that they behave in vivo as RA-dependent enhancers or inhibitors of RA-responsive genes, respectively. Their different activities on the RA signalling pathway might underlie the different responses of PML-RARα and PLZF-RARα APLs to RA treatment. The PLZF-RARα fusion protein contains an approximately 120-amino-acid N-terminal motif (called the POZ domain), which is also found in a variety of zinc finger proteins and a group of poxvirus proteins and which mediates protein-protein interactions. Deletion of the PLZF POZ domain partially abrogated the inhibitory effect of PLZF-RARα on RA-induced differentiation and on RA-mediated type II TGase up-regulation, suggesting that POZ-mediated protein interactions might be responsible for the inhibitory transcriptional activities of PLZF-RARα.

Acute promyelocytic leukemia (APL) is cytogenetically characterized by a reciprocal translocation that always involves chromosome 17, with a break within the locus encoding for the retinoic acid receptor α (RARα). Chromosome partners are chromosome 15, with the breakpoint located within the PML locus, or, less frequently, chromosome 11, with the breakpoint within the PLZF locus (15, 51). The hybrid genes so formed encode a PML-RARα or PLZF-RARα fusion protein, which retain equivalent portions of RARα (5, 10, 22, 24, 42, 43).

The 15;17 and 11;17 translocations are primary chromosome aberrations and are often the only cytogenetic anomalies in the neoplastic metaphases (37). Experimental evidence for leukemogenic potential, however, is available only for PML-RARα. PML-RARα blocks terminal differentiation of hematopoietic precursor cell lines in vitro (14, 16), and, in vivo, mice transgenic for PML-RARα manifest myeloid differentiative alterations with the phenotypic features of promyelocytic leukemia (2a, 18).

Despite the fact that PML-RARα and PLZF-RARα APLs are clinically indistinguishable, they differ in one important feature: their response to retinoic acid (RA). PML-RARα APL blasts are highly sensitive to RA, and most patients treated with RA achieve disease remission (9, 20, 33, 50). In contrast, PLZF-RARα-expressing APLs are not sensitive to RA treatment (13, 31).

The mechanisms through which PML-RARα blocks differentiation are not well understood. The cellular localization of PML-RARα is anomalous with respect to the corresponding wild-type proteins: RARα is nuclear diffuse and PML is localized within specific subnuclear structures called PML nuclear bodies, whereas PML-RARα has a distinct nuclear distribution pattern (microspeckled localization) (12, 27, 52). It was initially proposed that PML-RARα exerts a dominant-negative action on wild-type PML and RXR, an RARα cofactor (26, 53, 54), in that expression of PML-RARα provokes PML and RXR delocalization within microspeckles (12, 27, 44, 52). This hypothesis, however, has been recently challenged by the demonstration that delocalization of neither PML nor RXR is critical for the action of PML-RARα on differentiation (17). Nothing is known about the biological activity or cellular localization of the PLZF-RARα fusion protein.

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The molecular mechanisms of the APL response to RA remain unclear. Some investigators have suggested that the principal effect of RA is that it releases the dominant-negative effect of PML-RARα on wild-type PML or RXR, thereby removing the inhibitory activity on differentiation of the fusion protein. This hypothesis is supported by the fact that RA treatment of APL blasts induces progressive disappearance of the PML-RARα microspeckles and the reorganization of PML nuclear bodies and, as a consequence, restores the physiological localization of PML and RXR (12, 27, 52). Others instead propose that PML-RARα microspeckles are actively involved in conferring sensitivity to RA and hypothesize that PML-RARα exerts a direct effect on RA signalling in the presence of RA (2, 14, 15). The effects of RA on the PLZF-RARα protein are unknown, and the only available data refers to the in vitro transactivating activity of PLZF-RARα on cloned RA target genes, which appears to be indistinguishable from that of PML-RARα (6).

We expressed PLZF-RARα in various hematopoietic precursor cell lines and analyzed its effects on terminal differentiation and RA target genes, both in the absence and in the presence of pharmacological doses of RA, and compared the results with those obtained with PML-RARα in the same cell background. The comparison provided information essential for understanding the mechanisms at the basis of the differentiation block in APL blasts, as well as their response to RA.

MATERIALS AND METHODS

Preparation of PLZF, PLZF-RARα, and POZ-PLZF-RARα expression vectors. PLZF cDNA clones were isolated from a cDNA library of KG1 myeloid leukemia cells (Clontech, Palo Alto, Calif.) by using a DNA probe representative of the human PLZF POZ domain, according to standard screening protocols (46). The PLZF-POZ DNA probe was synthesized from total RNA from KG1 cells by reverse transcription-PCR with the PLZF1 (5′AAGCCTCATGCCTGAGCCGA3′) and PLZF2 (5′TACTCGATCTCCAGGATCTC3′) oligonucleotides. Of eight isolated cDNAs, one contained the full-length open reading frame of PLZF (clone 4B). It was 61 bp longer at the 5′ end than the reported PLZF B cDNA (5). The PLZF-RARα cDNA was reconstructed by PCR from wild-type PLZF and RARα cDNAs. Briefly, a PLZF fragment was amplified from position 1446 (corresponding to the *Sac*I site) (5′CGGGTGCGAGCTCGCGGGA3′) to position 1522 (corresponding to the PLZF-RARα crossover junction) (5′TTTGGCACCCCGGGAATGAGCCAGTA3′) so that a *Sma*I site (underlined) was inserted at the 3′ end of the PLZF fragment. A similar strategy was used to generate an RARα fragment from the PML junction point (5′ATCCCCAGCCCGGGTTGAGACCCAGA3′) to the *Sac*I site of RARα (5′TTGCGCACCTTCTCAATGAGCTC3′). The PLZF-RARα junction was then reconstructed by *Sma*I ligation of the two fragments. Since the *Sma*I site used to generate the PLZF-RARα junction introduced a novel amino acid, the appropriate junction was reconstructed by PCR-based mutagenesis (5′CGGGTGCGAGCTCGCGGGA3′, 5′CTCATTCCGCGATTGAGACCC3′, and 5′TTGCGCACCTTCTCAATGAGCTC3′). The newly generated PLZF-RARα fragment was then ligated to the appropriate RARα sequences to generate a full-length PLZF-RARα cDNA. To generate the POZ-PLZF-RARα mutant, the POZ domain-encoding sequence (nucleotides 1 to 471) of PLZF-RARα was replaced, by PCR, with a sequence encoding a translational initiation site. The 5′ oligonucleotide containing the translational initiation site was 5′AGATCCTGGAGATCGCCACCATGGAGGAACAGTGCCTGA3′. All of the DNA fragments generated by PCR were controlled by DNA sequence. The PLZF, PLZF-RARα, and POZ-PLZF-RARα cDNAs so obtained were subcloned into the pGMTSVneo Zn-inducible expression vector (14) and the adenovirus-based PMT2 expression vector (17). The preparation of the hemagglutinin-tagged PML-RARα expression vector has been already described (17).

Preparation of anti-PLZF antibodies. The PLZF open reading frame was cloned into the bacterial expression plasmid pGEX-2T after PCR-based creation of an in-frame *Bam*HI site corresponding to the first ATG of PLZF. Bacterial cultures expressing pGEX vectors were grown in Luria broth containing 50 μg of ampicillin per ml and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 to 6 h, and the induced bacteria were lysed by sonication in 1% Triton X-100. The glutathione *S*-transferase–PLZF fusion protein was purified by using glutathione-agarose and eluted with 15 mM glutathione. Anti-PLZF antibodies were prepared by immunizing New Zealand White rabbits with the purified glutathione *S*-transferase–PLZF fusion protein. The specificity of the anti-PLZF antibody was tested on COS-1 cells transiently transfected with a PLZF expression vector.

Cell culture, transfection, and Western blotting. U937 cells, HL60 cells, and the RA-resistant HL60 subclone (a gift from S. Collins) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. After electroporation, the cells were selected in G418 and subcloned under limiting-dilution conditions. Expression of the exogenous protein was evaluated by Western blotting after 6 to 12 h of induction with 100 μM $ZnSO_4$, using an anti-RARα-F (a gift from P. Chambon) or the anti-PLZF antibodies, according to established procedures.

Cell differentiation experiments and analysis of cell phenotype. Experiments involving differentiation of U937 cells under the influence of dihydroxy-vitamin D_3 (D3) and transforming growth factor β1 (TGF) were performed as described previously (14). The percentage of differentiation antigen-positive cells and fluorescence intensity were evaluated by FACScan (Becton Dickinson, Mountain View, Calif.) with appropriate antibodies (14, 17). RA-induced differentiation was investigated as previously described (14). For HL-60 and HL-60R cells, cells were incubated for 4 days in the presence of 10^{-6} M RA, 1.25% dimethyl sulfoxide (DMSO), or 250 ng of D3 per ml. Granulocytic and monocytic differentiation was evaluated by (i) flow cytometry analysis of differentiation-related surface antigens with appropriate antibodies (see Results) and (ii) light microscopy observation of May-Grünwald-Giemsa-stained samples.

Northern blot analysis and TGase assay. Total and poly(A)$^+$ RNA samples (10 μg each) were analyzed by Northern blotting according to established procedures (46). The RARβ probe was the *Sac*I/*Bam*HI restriction fragment of the recombinant PSG5 RARβ vector, the RARγ probe was the *Bam*HI restriction fragment of the recombinant PSG5 RARγ cDNA clone, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was the *Pst*I cDNA fragment of the rat GAPDH plasmid. TGase activity was measured with soluble cell fractions by measuring the incorporation of [^{3}H]putrescine (12.6 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) into casein in triplicate cultures, as previously described (2).

RA binding assays. COS-1 cells were transiently transfected by electroporation with a pSG5 expression vector for RARα or PML-RARα or with a pMT2 expression vector for PLZF-RARα. At 48 to 72 h after transfection, nuclear and cytosolic extracts were prepared from transfected COS-1 cells as previously described (39). For saturation binding and Scatchard analyses, nuclear extracts were incubated for 18 h at 4°C in the presence of increasing concentrations (0.01 to 20 nM) of ^{3}H-labelled all-*trans*-retinoic acid (t-RA) (50.7 Ci/mmol; DuPont/NEN, Boston, Mass.). Nonspecific binding activity was measured at each time point in the presence of a 200-fold molar excess of unlabeled t-RA. Specific ligand binding to receptors was separated from free radioactivity by using PD10 desalting columns (Pharmacia, Uppsala, Sweden) as described previously (21). Linear least-squares analysis of the Scatchard plot was performed with the aid of the computer program BDATA-EMF.

RESULTS

Expression of PLZF-RARα blocks monocytic and granulocytic differentiation of U937 and HL-60 precursor cell lines induced by D3-TGF or DMSO. To test whether PLZF-RARα blocks terminal differentiation in vitro, the PLZF-RARα cDNA was expressed in the HL-60 and U937 hematopoietic precursor cell lines, and their potential to undergo terminal differentiation was analyzed. The promonocytic U937 cells proceed towards terminal differentiation under the influence of 250 ng of D3 per ml and 1 ng of TGF per ml (48). The myeloblastic HL-60 cells differentiate towards the granulocytic line when induced with DMSO and towards the monocytic line when stimulated with the combination of D3 and TGF (7, 48).

The PLZF-RARα cDNA under the control of the Zn-inducible MT-1 promoter was electroporated into U937 and HL-60 cells. After G418 selection and limiting dilution, 8 of 15 U937 clones and 5 of 12 HL-60 clones were positive for PLZF-RARα expression by Western blotting analysis with anti-PLZF (see Materials and Methods) or anti-RARα antibodies. Two clones of each cell type were selected for further analysis by two criteria: (i) increased protein expression upon Zn treatment (Fig. 1A) and (ii) capacity to undergo terminal differentiation in the absence of Zn (not shown). All of the U937 clones that were analyzed revealed a slight PLZF-RARα expression in the absence of Zn induction (Fig. 1A); however, this level of Zn-independent fusion protein expression did not interfere significantly with differentiation induced by D3-TGF (not shown). Clones transfected with the empty expression vector were used as controls.

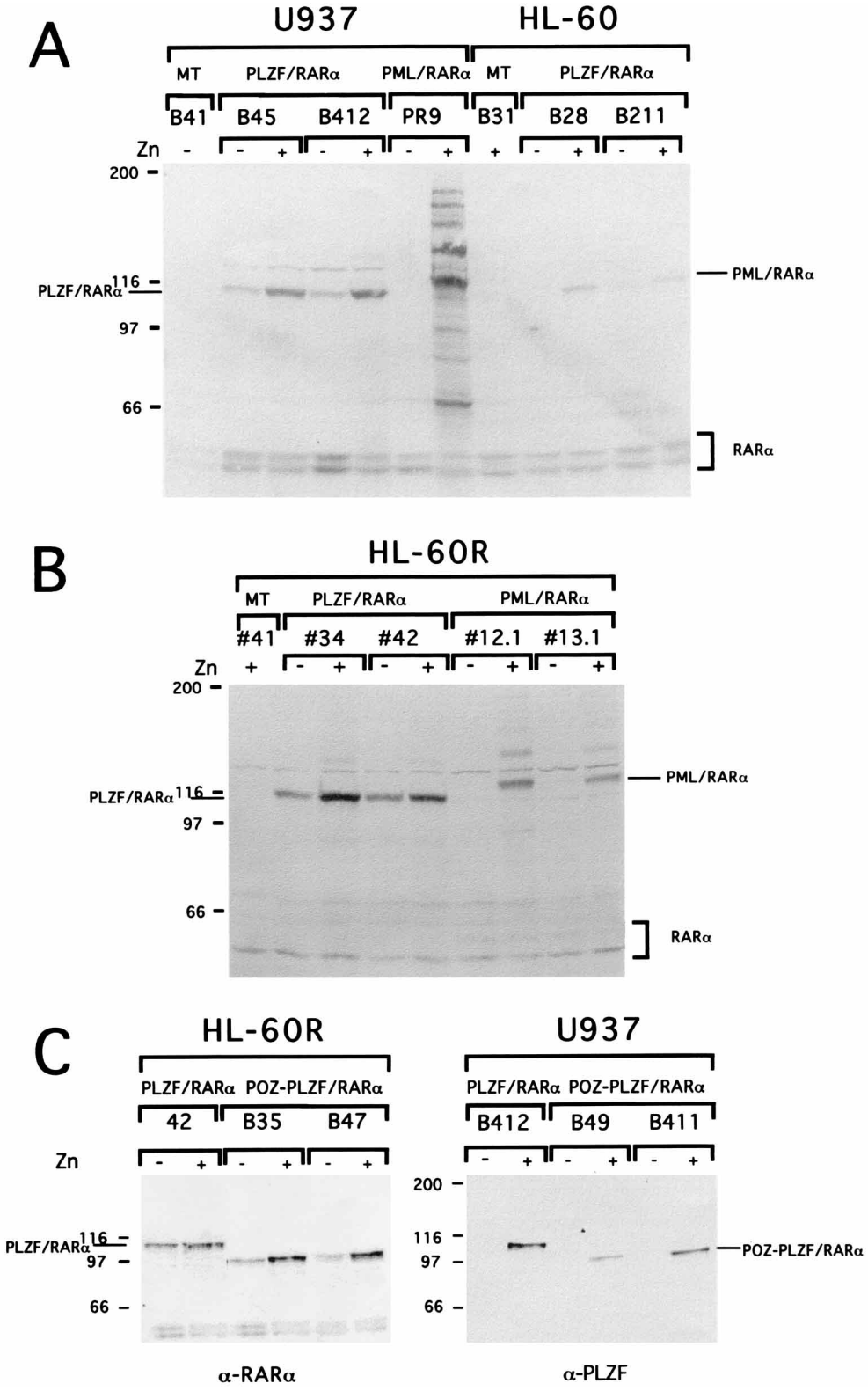

FIG. 1. Western blot analysis of expression of PLZF-RARα and PML-RARα (A and B) and POZ-PLZF-RARα (C) in U937, HL-60, and HL-60R cells. The proteins, cell lines, and clones are indicated above the blots. Molecular weight markers (in thousands) are given to the left of each blot. Each lane was loaded with lysates from 2×10^5 cells grown in the presence (+) or absence (−) of Zn induction. Control cells were transfected with the empty pMT expression vector (MT). The Western blots were probed with anti-RARα (αRARα) or anti-PLZF antibodies, as indicated.

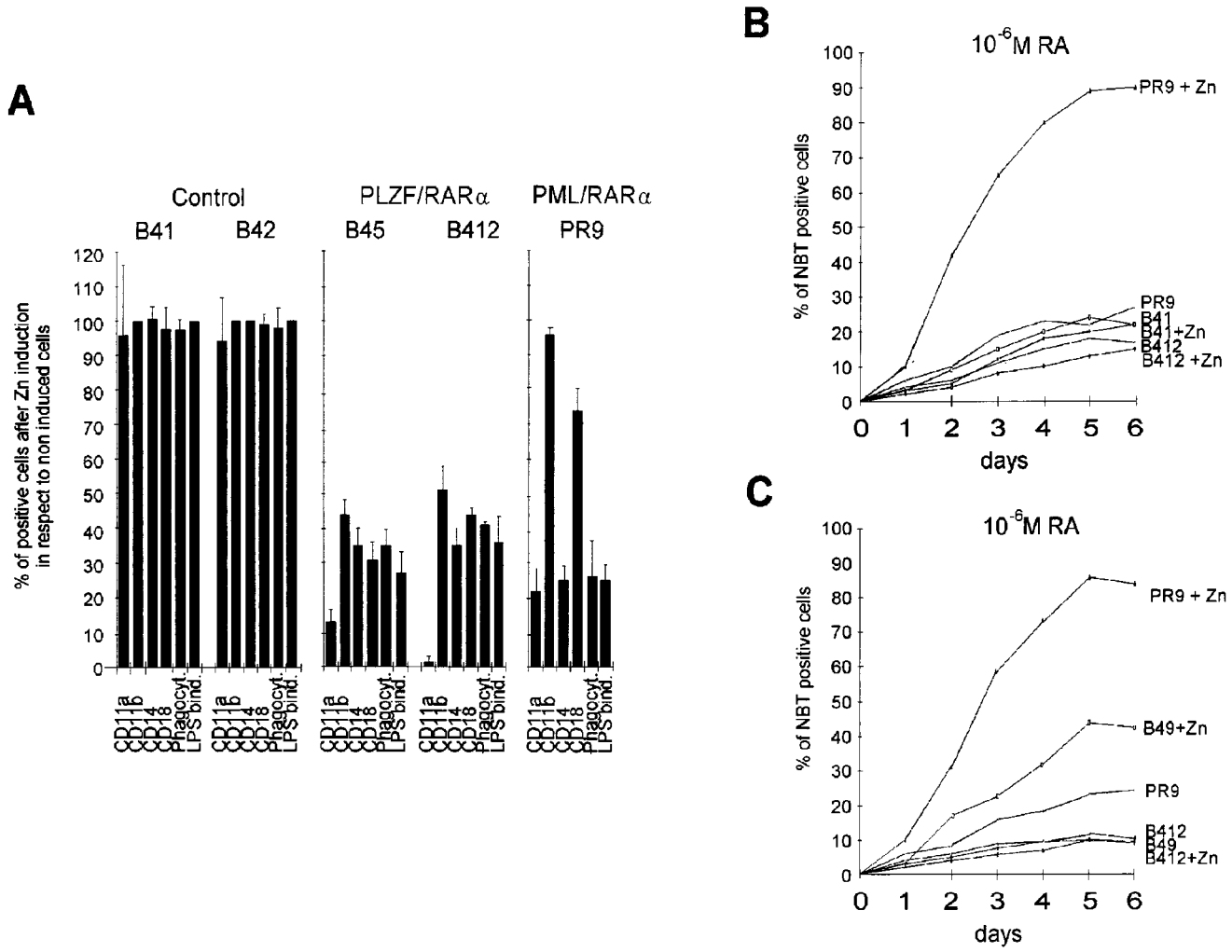

FIG. 2. Differentiation levels induced by D3-TGF (A) or RA (B and C) in U937 cells transduced with the MT vector (control B41 clone) or overexpressing PLZF-RARα (B412 clone), PML-RARα (PR9 clone), or POZ-PLZF-RARa (B49 clone) proteins. (A) D3-TGF-induced differentiation was measured by quantitative expression of CD11a, CD11b, CD14, and CD18 antigens and by the percentages of cells with phagocytic activity and capacity to bind LPS in the absence and in the presence of Zn treatment. The results obtained after zinc-induced expression of the exogenous proteins are expressed as percentages of the values obtained in the absence of zinc. Three experiments for each clone were performed, and standard deviations are given. (B and C) RA-induced differentiation was measured by the NBT reduction test. Each experiment is representative of three that gave similar results.

Terminal differentiation was induced by 4 days of treatment with D3-TGF (U937 and HL-60 cells) or DMSO (HL-60 cells) in the presence or absence of Zn and monitored by quantitative fluorescence-activated cell sorter analysis of appropriate surface differentiation antigens (CD11a, CD11b, CD14, CD18, phagocytic activity, and lipopolysaccharide [LPS] binding in U937 cells treated with D3-TGF; CD11a, CD11b, CD14, CD18, and CD54 in HL60 cells treated with D3-TGF; and CD11a, CD11b, and CD18 in HL60 cells treated with DMSO). Expression of all of these markers was low or absent in the unstimulated parental cell lines or in the non-Zn-treated clones and increased progressively during differentiation (not shown), as previously reported (14, 17, 48). To avoid biases of clonality and culture condition variations, differentiation was evaluated for each clone in both the presence and absence of Zn-induced protein expression. Results are given as the percentage of differentiation marker expression in the presence of Zn with respect to expression in its absence. The differentiation-related increase in differentiation marker expression was not affected by Zn treatment in either HL-60 or U937 cells (Fig. 2A, control).

The effects of PLZF-RARα on the monocytic differentiation of U937 cells induced by D3-TGF are reported in Fig. 2A. Both the B45 and B412 PLZF-RARα-expressing clones differentiated poorly in response to D3-TGF after Zn-induced PLZF-RARα expression. The differentiative block mediated by PLZF-RARα was equal (CD14 antigen, phagocytic activity, and LPS binding) or superior (CD11a, CD11b, and CD18 antigens) to that obtained by expressing PML-RARα in the same cells (Fig. 2A, clone PR9).

The effects of PLZF-RARα expression on D3-TGF-induced monocytic and DMSO-induced granulocytic differentiation of HL-60 cells are shown in Fig. 3. Zn-induced PLZF-RARα expression impaired the capacity of the B28 and B211 clones to undergo D3-TGF- or DMSO-induced differentiation, as revealed by differentiation antigen analysis. Similar results were obtained by morphological monitoring of differentiation: D3-TGF- or DMSO-treated B28 and B211 cells maintained their

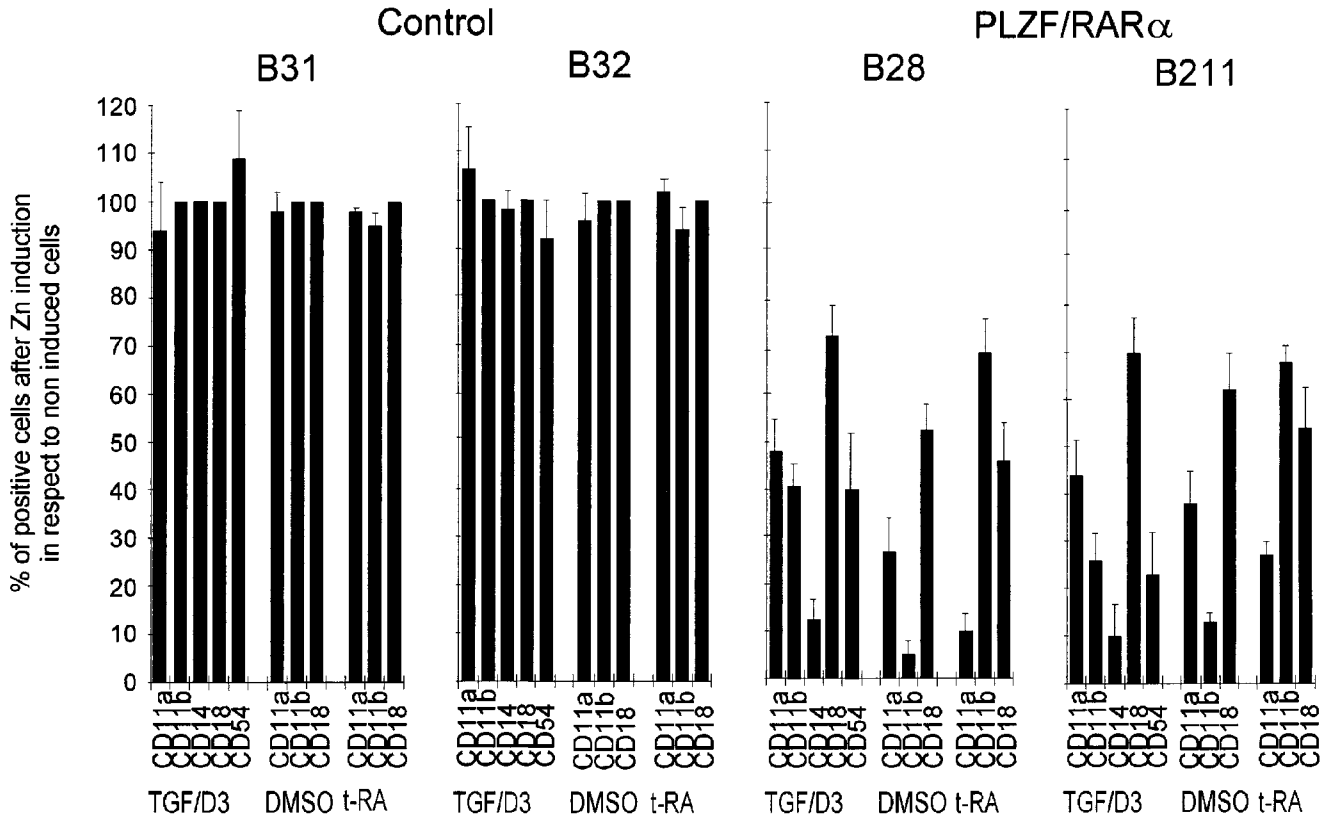

FIG. 3. Differentiation levels induced by D3-TGF, DMSO, or RA in HL-60 cells transduced with the MT vector (B31 and B32 clones) or overexpressing PLZF-RARα (B28 and B211 clones). Differentiation was measured by quantitative expression of CD11a, CD11b, CD14, CD18, and CD54 antigens for D3-TGF-treated cells and of CD11a, CD11b, and CD18 antigens for DMSO- and RA-treated cells. Results are expressed as described in the legend to Fig. 2. Three experiments for each clone were performed, and standard deviations are given.

blast-like morphology in the presence of Zn-induced PLZF-RARα expression, while they acquired typical monocytic or granulocytic features, respectively, in the absence of Zn (data not shown). The capacity of PLZF-RARα to block terminal differentiation of HL-60 cells could not be compared with that of PML-RARα because HL-60 cells are not permissive for PML-RARα expression (14).

These results indicate that PLZF-RARα expression blocks monocytic and granulocytic terminal differentiation of hematopoietic precursor cell lines and suggest that this biological activity of PLZF-RARα, as in the case of PML-RARα, contributes to its in vivo leukemogenetic potential.

PLZF-RARα and PML-RARα have different effects on the RA-induced monocytic or granulocytic differentiation of U937 and HL-60 cells. We have previously demonstrated that expression of PML-RARα increases the sensitivity of U937 cells to the differentiative action of RA (14). The effect of PLZF-RARα on RA-induced differentiation was therefore investigated by analyzing the RA responses of U937 and HL-60 cells expressing PLZF-RARα. U937 cells differentiate poorly in response to RA, as determined by the percentage of differentiated cells (between 20 and 50%) and the degree of differentiation (no modulation of the conventional differentiation antigens) (40). RA-induced differentiation of U937 cells is usually monitored by measuring the capacity of differentiated cells to reduce nitroblue tetrazolium (NBT). The B41 control clone differentiated poorly after 6 days of RA treatment, and differentiation was not affected by Zn treatment (15 and 22% NBT-positive cells with and without Zn, respectively) (Fig. 2B). In the PML-RARα-transduced cells (PR9 clone), RA induced a comparable low degree of differentiation when cells were grown in the absence of Zn (20% NBT-positive cells), while differentiation was almost complete (approximately 90% NBT positivity) when the same cells were induced by RA in the presence of Zn. The response to RA was, instead, modest in the B412 PLZF-RARα-transfected clone in both the presence and absence of Zn-induced PLZF-RARα expression (15 and 23% NBT-positive cells, respectively) (Fig. 2B). Comparable results were obtained by using another PLZF-RARα U937 clone (clone B45; data not shown).

Granulocytic differentiation of HL-60 cells by RA can be monitored by analyzing surface differentiation antigens (CD11a, CD11b, and CD18) or by morphological evaluation. PLZF-RARα expression blocked RA-induced differentiation, as evaluated by either of these criteria (see Fig. 3 for surface marker analysis; morphology not shown).

In summary, it appears that PLZF-RARα expression blocks RA-induced differentiation. The effect seems to differ from that of PML-RARα, which, at least in the U937 cells, increases the RA response. These data suggest that PML-RARα and PLZF-RARα exert different effects on the RA signalling pathway. Direct proof for this proposal was searched for by testing the capacities of PML-RARα and PLZF-RARα to restore sensitivity to RA in RA-resistant hematopoietic cells.

PML-RARα, but not PLZF-RARα, restores the sensitivity to RA of an RA-resistant HL-60 subline. HL-60R is an HL-60 subline selected for its ability to grow in the presence of RA (8). Resistance is associated with a point mutation in the

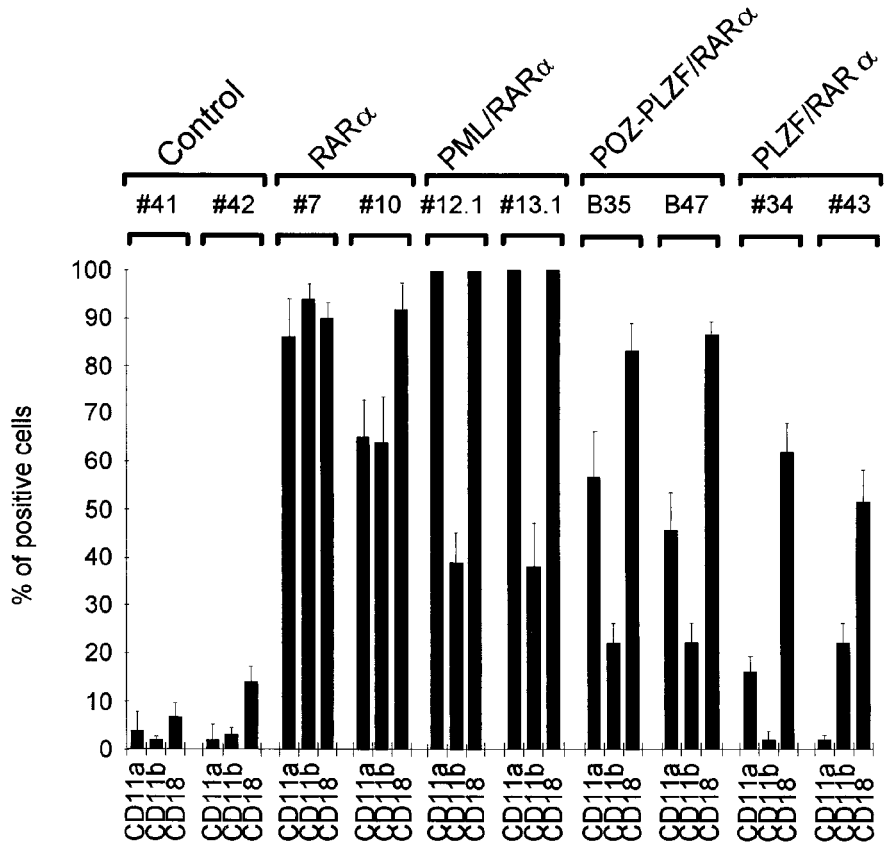

FIG. 4. Differentiation levels induced by RA in HL-60R cells transduced with the MT vector (clones 41 and 42) or overexpressing RARα (clones 7 and 10), PML-RARα (clones 12.1 and 13.1), PLZF-RARα (clones 34 and 43), or POZ-PLZF-RARa (clones B35 and B47). Differentiation was measured by quantitative expression of CD11a, CD11b, and CD18 antigens. Results are expressed as percentages of positive cells after RA treatment in the presence of Zn induction. Three experiments for each clone were performed, and standard deviations are given.

RARα ligand binding domain that introduces a frameshift and results in premature termination of the receptor (45). Reintroduction of wild-type RARα in these cells restores sensitivity to RA (8). PML-RARα, PLZF-RARα, and RARα (as a control) cDNAs were transfected into HL-60R cells by using the pGMTSVneo expression vector. Two clones that expressed high levels of exogenous protein expression after Zn induction were selected from each transfection (see Fig. 1B for PLZF-RARα and PML-RARα protein expression results). Two clones transfected with the empty expression vector were used as controls (clones 41 and 42). It should be noted that HL-60R cells, like the parental HL-60 cells, are not permissive for PML-RARα expression (14) and that we obtained HL-60R (but not HL-60) PML-RARα-expressing clones by the concomitant expression of bcl-2. The mechanism(s) by which bcl2 makes HL-60R cells permissive for PML-RARα expression will be reported elsewhere. Parallel control experiments revealed that bcl2 expression does not interfere with the RA sensitivity of HL-60R cells (not shown). RA treatment of HL-60R control clones neither modified their growth potential (not shown) nor induced differentiation, as determined by surface marker analysis (Fig. 4, HL-60R clones 41 and 42) and morphological analysis (not shown). RARα expression restored RA sensitivity of HL-60R cells, as demonstrated by the facts that cells stopped growing (not shown) and acquired the ability to fully express differentiation markers (Fig. 4, clones 7 and 10) and the morphological features of differentiated cells (not shown) when treated with RA. Likewise, expression of PML-RARα largely restored RA sensitivity of HL-60R cells (Fig. 4, clones 12.1 and 13.1), whereas PLZF-RARα did not, except for a mild stimulatory effect on CD18 expression (Fig. 4, clones 34 and 43).

PML-RARα and PLZF-RARα have similar RA binding affinities and opposite effects on RA target genes in vivo. To investigate the mechanisms underlying the opposite effects of PML-RARα and PLZF-RARα on RA-mediated differentiation, we evaluated their capacities to bind RA and to activate RA target genes in vivo.

Nuclear extracts from either COS-1 cells transiently transfected with PLZF-RARα or U937 B45 clone stably expressing PLZF-RARα were labelled for 18 h with increasing concentrations of [^{3}H]t-RA and analyzed by using PD10 desalting columns (see Materials and Methods). The binding affinity of t-RA to PLZF-RARα was analyzed directly by saturation kinetics and Scatchard analysis and compared to those for PML-RARα and RARα (39). The t-RA binding to PLZF-RARα was specific and saturable (not shown). Scatchard analysis of this binding indicated a single high-affinity binding site on PLZF-RARα expressed by either COS-1 or U937 cells (not shown). The calculated dissociation constants were 0.17 and 0.19 nM, respectively, and were similar to those determined for RARα and PML-RARα (Table 1).

U937 cells expressing PML-RARα or PLZF-RARα were then analyzed for the effects of RA on four RA target genes (those for RARβ, RARγ, CRABP1, and CRABP2) (25, 35, 38, 47) and on the type II TGase gene, a gene that is regulated by

TABLE 1. Dissociation constants for the binding of [^{3}H]t-RA to human RARα, PML-RARα, or PLZF-RARα

Transfected cell line	RA-binding protein	Dissociation constant (nM)[a]
COS-1	RARα	0.09 ± 0.03
	PML-RARα	0.13 ± 0.02
	PLZF-RARα	0.17 ± 0.01
U937	PLZF-RARα	0.19 ± 0.06

[a] Dissociation constants were determined by Scatchard analysis. Numbers represents the means ± standard deviations for at least three individual experiments.

retinoids and was recently shown to be a marker of RA-induced differentiation in APL cells (2).

Type II TGase was assessed by measuring cytosolic TGase activity in U937 control cells (clone B41) and in PML-RARα (clone PR9)- and PLZF-RARα (clone B412)-expressing cells before RA treatment and after 24 and 48 h in the presence or absence of RA (Fig. 5A). A two- to threefold increase of TGase activity was observed in B41 control cells after 48 h of RA treatment, irrespective of Zn treatment. In PR9 cells induced to express PML-RARα, TGase activity was strongly up-regulated after 24 and 48 h of RA treatment (5- to 10-fold increase over that in non-Zn-induced cells). In contrast, in PLZF-RARα-expressing cells there was no induction after 24 h and a slight down-regulation of TGase activity after 48 h of RA treatment (Fig. 5A).

RARγ expression was evaluated by Northern blot analysis of poly(A)$^+$ mRNA from U937 control cells (clone B41) or cells expressing PML-RARα (clone PR9) or PLZF-RARα (clone B412), with the full-length RARγ cDNA as a probe. The results revealed a slight up-regulation of the RARγ transcript in the control B41 cells after 24 h of RA treatment and disappearance of the signal after 48 h. Instead, a strong induction of RARγ expression was detected in PML-RARα-expressing cells after 24 h, with a decline in the hybridization signal after 48 h. There was no, or very little, RARγ expression in PLZF-RARα-expressing cells after either 24 or 48 h (Fig. 5B). The approximately 3.5-kb RARγ-hybridizing transcript in the RNA samples from PLZF-RARα-expressing cells derives from the cross-hybridization of the RARγ probe with the PLZF-RARα transcript, as demonstrated by the hybridization of the same blot with an RARα cDNA probe (data not shown).

Total RNA from the same U937 clones was also analyzed for RARβ expression (Fig. 5C). Since we have noted a great variability in the degree of RARβ expression after RA treatment of different U937 clones (not shown), to avoid biases from clonal variability, the RARβ response to RA was compared for the same clones in the presence or absence of Zn induction. Expression of PML-RARα resulted in increased accumulation of RARβ transcripts after 24 h of RA treatment, as compared to that in non-Zn-treated cells, while PLZF-RARα expression did not affect RARβ expression at the same time point after RA treatment (Fig. 5C). CRABP1 and CRABP2 genes were not detected in U937 cells under any culture condition (data not shown).

It appears that, in vivo, PML-RARα potentiates the RA response of TGase and the two tested RA target genes (RARβ and RARγ), while PLZF-RARα either exerts no effect on (RARβ) or inhibits (TGase and RARγ) RA target gene activation.

Deletion of the POZ domain partially releases the inhibitory effect of PLZF-RARα on RA signalling. The PLZF-RARα fusion protein retains the POZ domain of PLZF (5). The POZ domain is a 120-amino-acid conserved motif present in a large family of proteins that includes a variety of zinc finger proteins and a group of poxvirus proteins (1). The POZ domains from the zinc finger proteins ZID, Ttk, and GAGA have been shown to function as protein interaction domains and to inhibit the interaction of their associated DNA binding regions with DNA (1). To preliminarily investigate the mechanism(s) through which the fusion of PLZF with RARα results in a protein with inhibitory activity on the RA-dependent signalling pathway, we analyzed the effects of the deletion of the POZ domain on the capacity of PLZF-RARα to block RA differentiation and to repress RA target genes in vivo.

A PLZF-RARα mutant with a deletion of the region encoding the PLZF POZ domain (POZ-PLZF-RARα; see Materials and Methods) was cloned under the control of the Zn-inducible MT-1 promoter and electroporated into U937 and HL-60R cells. Expression of the POZ-PLZF-RARα polypeptide was analyzed in individual clones by Western blotting with anti-PLZF antibodies. Two clones each of U937 (B49 and B411) and HL60R (B35 and B47) cells were chosen for further analysis based on their levels of exogenous protein expression (Fig. 1C). Immunofluorescence analysis with anti-PLZF antibodies revealed that the POZ-PLZF-RARα polypeptide had the same microsplecked localization as PLZF-RARα (data not shown).

We then compared the capacities of U937 and HL60R cells expressing POZ-PLZF-RARα, PLZF-RARα, or PML-RARα to differentiate in the presence of RA. In the absence of Zn treatment, RA induced a similarly low degree of differentiation (10 to 20% NBT-positive cells) of U937 cells expressing POZ-PLZF-RARα, PLZF-RARα, or PML-RARα. However, upon Zn induction of exogenous protein expression, RA-induced differentiation increased to approximately 40 to 50% in the POZ-PLZF-RARα U937 cells, a level which was intermediate between those of PLZF-RARα (approximately 20%) and PML-RARα (approximately 90%) cells (Fig. 2C). Similar results were obtained with HL60R cells: RA treatment of POZ-PLZF-RARα-expressing HL60R cells induced partial up-regulation of the CD11a, CD11b, and CD18 differentiation antigens (Fig. 4).

U937 cells expressing POZ-PLZF-RARα were then analyzed for the effects of RA on TGase and RARγ. In PR9 cells induced to express POZ-PLZF-RARα, TGase activity was up-regulated after 24 and 48 h of RA treatment (three- to fourfold increase over that in non-Zn-induced cells) (Fig. 5A). In contrast, there was no, or very little, RARγ expression in POZ-PLZF-RARα-expressing cells after either 24 or 48 h of RA treatment (Fig. 5B).

In summary, the analysis of the sensitivity of U937 and HL60R cells expressing POZ-PLZF-RARα to the differentiative action of RA revealed that the POZ-PLZF-RARα mutant is able to mediate RA sensitivity, although to a lesser extent than PML-RARα, therefore suggesting that the deletion of the POZ domain partially releases the inhibitory effect of PLZF-RARα. However, analysis of the capacity of the POZ-PLZF-RARα protein to activate RA target genes in vivo yielded heterogeneous results: this protein potentiated the RA response of TGases, while it exerted no effect on RARγ gene activation.

DISCUSSION

PML-RARα and PLZF-RARα are the abnormal protein products of APLs with either t(15;17) or t(11;17). These two APL types are phenotypically indistinguishable, from the point of view of both the morphology of the leukemic blasts and the

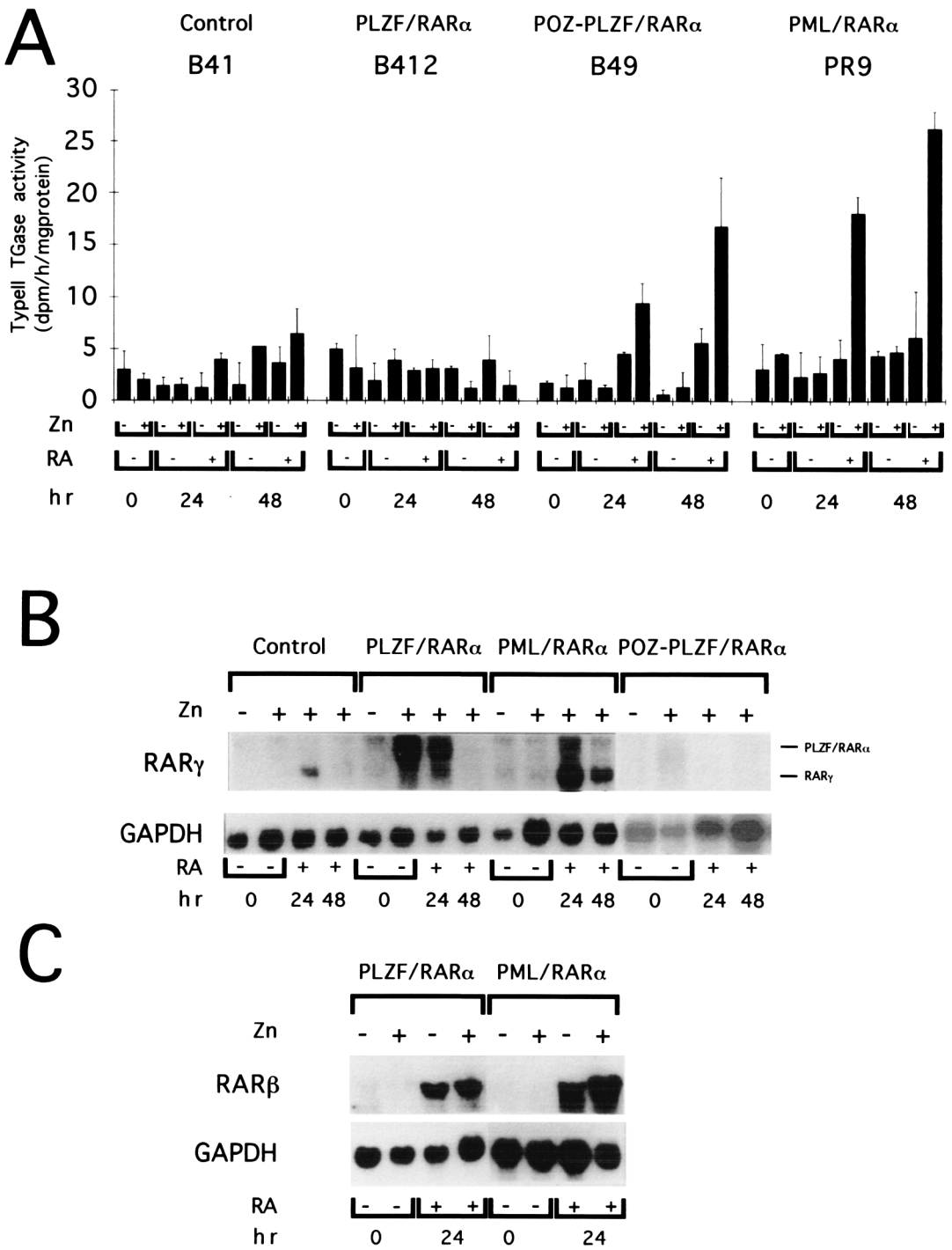

FIG. 5. RA-induced modulation of type II TGase activity and RARγ and RARβ expression in control U937 cells (B41 clone) and U937 cells expressing PLZF-RARα (B412 clone), POZ-PLZF-RARα (B49 clone), or PML-RARα (PR9 clone). The various cell samples were analyzed in the presence (+) or absence (−) of Zn induction before (time zero) or 24 or 48 h after RA (+) or ethanol (−) treatment. (A) TGase activity. Results are the means ± standard deviations for triplicate samples from a single experiment. This experiment is representative of three that gave similar results. (B and C) Northern blotting analysis of RARγ (B) and RARβ (C) expression. Ten micrograms of poly(A)$^+$ mRNA (B) or total mRNA (C) from the indicated samples was hybridized with an RARγ (B) or RARβ (C) cDNA probe. The same filters were hybridized with a GAPDH cDNA probe.

clinical presentation of the disease. Therapeutically, however, they differ in that the type that expresses PML-RARα responds to RA treatment, whereas the type that expresses PLZF-RARα does not (15, 50, 51).

In the absence of pharmacological doses of RA, the biological activity of PLZF-RARα on the terminal differentiation of hematopoietic precursors is similar to that of PML-RARα. PLZF-RARα blocks D3-induced monocytic differentiation to the same extent as PML-RARα. PLZF-RARα was also demonstrated to block DMSO-stimulated granulocytic differentia-

tion in the myeloblastic HL-60 cell line. We have recently found that PLZF-RARα blocks the granulocyte colony-stimulating factor-induced granulocytic-erythroid differentiation of the multipotent 32-D cell line (unpublished results). No such information is available for the PML-RARα fusion protein, as its expression induces death of HL-60, 32-D, and other precursor cell lines (11a). It therefore appears that PLZF-RARα is able to block multilineage myeloid differentiation triggered by different agents (D3-TGF, DMSO, and granulocyte colony-stimulating factor) with seemingly different mechanisms of action.

When the effects of the two fusion proteins on the RA differentiative response are examined, PLZF-RARα and PML-RARα are found to behave differently. We have previously shown that PML-RARα enhances the RA response of cells that respond poorly to RA (U937 cells) (14). We now extend this observation to RA-resistant cells and show that PML-RARα is also able to confer sensitivity to RA to these cells. In contrast, PLZF-RARα is not able to do so in RA-resistant cells, and it inhibits the RA response of both poorly (U937) and highly (HL-60) responsive cells. The sensitivity of promyelocytic blasts to the action of RA therefore seems to strictly depend on the type of fusion protein present.

Even though the biological effects of PML-RARα and PLZF-RARα on the cell response to RA are different, at the molecular level both fusion proteins retain the same portion of RARα, from the B to the F domain (5, 43). A convincing hypothesis would be that the response of promyelocytic blasts to the action of RA is the direct consequence of PML-RARα or PLZF-RARα activity on the RA signalling pathway. Our data are consistent with the proposal that pharmacological doses of RA induce PML-RARα, but not PLZF-RARα, to act as an activator on RA-responsive genes. We studied the response to RA of three RA target genes (those for TGase, RARβ, and RARγ) in U937 cells in the presence and absence of PML-RARα or PLZF-RARα and found that the response was enhanced by PML-RARα but was unaffected or repressed by PLZF-RARα. It is somewhat surprising that PLZF-RARα and PML-RARα regulate RA target genes differently in vivo, seeing that PML-RARα and PLZF-RARα have been demonstrated to have analogous transactivating properties on cloned RA-responsive reporter genes in transient-transfection experiments (6). Different regulation of cloned and native promoters might explain this discrepancy.

The mechanism through which the PML-RARα fusion results in a protein with high RA-dependent enhancer activity on RA target genes in vivo, while the PLZF-RARα fusion does not, remains unclear. The distinct transcriptional properties of the fusion proteins might derive from their different abilities to bind DNA, RA, or a variety of nuclear factors that physiologically form complexes with RARs (RXRs, corepressors, and co-activators) (36). RARs are ligand-dependent transcription factors that activate transcription through specific DNA binding sites (RA-responsive elements [RAREs]) in their target genes. RARs bind RA target gene promoters in physical association with RXRs, a family of retinoid receptors which act as cofactors for several other nuclear receptors (3, 34). In the absence of RA, corepressors are associated with the RAR-RXR heterodimer, thus determining transcriptional repression (4, 19, 28). RA:RAR-RXR interaction induces the dissociation of the corepressors and the recruitment of coactivators, resulting in a transcriptionally active complex (23, 29, 30, 41, 49). Both PML-RARα and PLZF-RARα retain most of the RARα protein and thus the potential to bind DNA, RA, and the various RAR-RXR cofactors. Indeed, both fusion proteins are able to bind to RAREs, to transactivate RA target genes in vitro (data not shown and references 10, 22, 24, 32, and 42), and to bind RXRs (11, 32, 44) and RA (39). The binding of PLZF-RARα to distinct RAREs is slightly different from that of PML-RARα, thereby suggesting that the two fusion proteins may have different target gene specificities (11). The capacity of PML-RARα and PLZF-RARα to bind to RAR-RXR coactivators and corepressors has yet to be defined. However, since these interactions were mapped to RARα regions which are retained within both fusion proteins (the RAR hinge region and AF-2 domain, respectively), they are unlikely to account for the differences between PML-RARα and PLZF-RARα.

Alternative explanations can be envisioned. The different regulation of RA target genes by PML-RARα and PLZF-RARα might result from differential recruitment of nuclear factors through the PML or PLZF moiety of the fusion protein. They both contain unique protein-protein dimerization interfaces: the PML coiled-coil region, which contains four clusters of heptad repeats with hydrophobic amino acids at the first, fourth, and eighth positions (15), and the PLZF POZ domain, an approximately 120-amino-acid motif which characterizes a subfamily of C2-H2 zinc finger proteins (11). The coiled-coil region and the POZ domain are involved in the formation of PML-RARα (39) and PLZF-RARα (our unpublished results) high-molecular-weight nuclear complexes, respectively, and their integrity is crucial for the biological activity of both fusion proteins. Deletion of the PML coiled-coil region abrogates the capacity of PML-RARα to block D3-induced differentiation and to increase RA sensitivity (17), while deletion of the POZ domain partially releases the inhibitory effect of PLZF-RARα on the RA-induced differentiation and RA-mediated activation of TGase. While the proteins that interact with the PML coiled-coil region and PLZF POZ domain are not entirely known, each of the two domains is capable of forming homo-oligomers. The PML coiled-coil region is involved in the formation of PML-RARα homodimers and PML-RARα–PML heterodimers (24, 44), whereas the PLZF POZ domain is involved in the formation of PLZF-RARα homodimers and PLZF-RARα–PLZF heterodimers (11). Of note, we have recently identified PML-RARα mutants that retain the capacity to form PML-RARα homodimers but have lost the ability to form heterodimers with PML (17). Functional analysis of these mutants in U937 cells revealed that the competence to increase RA sensitivity correlates with that of forming PML-RARα homodimers (17). Thus, the PML-RARα homodimers might have a more potent RA-dependent enhancer function than, in myeloid cells, the physiological RARα-RXR heterodimer (26, 53, 54). The inhibitory activity of PLZF-RARα on RA target genes might instead be due, in part, to its interaction with POZ-binding proteins with the ability to silence transcription. The identification of POZ-binding proteins that interact with PLZF and PLZF-RARα might help in clarifying this issue.

In conclusion, we demonstrated that PML-RARα and PLZF-RARα have similar inhibitory effects on terminal differentiation of hematopoietic precursor cells in the absence of RA, while they have opposite effects on RA differentiation and regulation of RA target genes in the presence of pharmacological doses of RA. These results might explain why PLZF-RARα and PML-RARα leukemias have identical phenotypes but opposite sensitivities to RA treatment.

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