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Genetics

PLZF–RARα fusion proteins generated from the variant t(11;17)(q23;q21) translocation in acute promyelocytic leukemia inhibit ligand-dependent transactivation of wild-type retinoic acid receptors

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ABSTRACT **Recently, we described a recurrent variant translocation, t(11;17)(q23;q21), in acute promyelocytic leukemia (APL) which juxtaposes *PLZF*, a gene encoding a zinc finger protein, to *RARA*, encoding retinoic acid receptor α (RARα). We have now cloned cDNAs encoding PLZF–RARα chimeric proteins and studied their transactivating activities. In transient-expression assays, both the PLZF(A)–RARα and PLZF(B)–RARα fusion proteins like the PML–RARα protein resulting from the well-known t(15;17) translocation in APL, antagonized endogenous and transfected wild-type RARα in the presence of retinoic acid. Cotransfection assays showed that a significant repression of RARα transactivation activity was obtained even with a very low PLZF–RARα-expressing plasmid concentration. A "dominant negative" effect was observed when PLZF–RARα fusion proteins were cotransfected with vectors expressing RARα and retinoid X receptor α (RXRα). These abnormal transactivation properties observed in retinoic acid-sensitive myeloid cells strongly implicate the PLZF–RARα fusion proteins in the molecular pathogenesis of APL.**

Nonrandom chromosomal translocations play an important role in the pathogenesis of human malignant hemopathies (1, 2). Acute promyelocytic leukemia (APL) is a malignant proliferation of cells blocked at the stage of promyelocytic differentiation and characterized by the specific reciprocal chromosomal translocation t(15;17) observed in >95% of patients (3). The t(15;17) juxtaposes the retinoic acid receptor α (RARα) gene, *RARA*, to a zinc finger gene, *PML* (for promyelocytic leukemia), which is normally located on chromosome 15 (4–16). Two reciprocal fusion genes are generated: *PML–RARA* on der(15) and *RARA–PML* on der (17), with corresponding mRNA transcripts and protein products. These interesting albeit paradoxical findings prompted speculation on the possible relationship between the clinical effect of all-*trans*-retinoic acid (RA) on APL (17–19) and the presence of the abnormal receptor. An important question has been raised concerning the role that the PML–RARα fusion protein may play in the molecular pathogenesis of APL: whether the PML–RARα antagonizes the wild-type RARα (or other RARs) or interferes with the normal function of the wild-type PML protein, or both? Previous transient-expression studies showed that PML–RARα behaves differently from wild-type RARα in HL-60 promyelocytic leukemia cells and some nonhematopoietic cell lines (8–10, 13), suggesting that the fusion receptor could antagonize the normal RARα-regulated differentiation pathway.

While studying the molecular characteristics of a large series of Chinese APL patients, we identified (20, 21) a variant reciprocal chromosomal translocation, t(11;17)(q23;q21). Molecular studies allowed us to identify a fusion between the *RARA* and another zinc finger gene, *PLZF* (for promyelocytic leukemia zinc finger). Recently, three other APL cases with t(11;17) have been encountered in Caucasian APL. Molecular analysis determined that these cases harbored the *PLZF–RARA* fusion transcript described in the Chinese case (S.W., C.C., A.Z., S.-J.C. and Z.C., unpublished work). In contrast to the t(15;17) APL, the t(11;17) APL cases respond poorly if at all to RA (unpublished data). The t(11;17) is therefore a recurrent albeit rare event in malignant transformation that appears clinically different from the common t(15;17) APL. Structural and functional studies of the chimeric receptor PLZF–RARα, and comparison with PML–RARα, may give further insight into the pathogenesis of APL and help to identify the determinant molecules underlying the efficacy of differentiation therapy by RA in APL.

We have cloned the *PLZF–RARA* hybrid genes and carried out a functional study on the transactivation properties of the PLZF–RARα fusion receptors. We show that the two PLZF–RARα isoforms, like PML–RARα, function as "dominant negative" mutants against wild-type RARα in a number of cell lines, including RA-sensitive human myeloid cells.

MATERIALS AND METHODS

Expression Vector and Reporter Gene Constructs. Total RNA was extracted by a one-step guanidinium thiocyanate/phenol/chloroform method (22) from the cells of the index patient with t(11;17) (20, 21). *PLZF(A)–RARA*, *PLZF(B)–RARA* and *RARA1–PLZF* chimeric cDNAs were amplified by PCR with the *Pyrococcus furiosus* (*Pfu*) DNA polymerase (Stratagene) using oligonucleotides up- and downstream of the open reading frame (ORF) (20, 23, 24). The amplified cDNAs were subjected to nucleotide sequence analysis and cloned into the *Eco*RI site of the expression vector pSG5. For control purpose, truncated *PLZF(B)* cDNAs lacking the sequence encoding the last seven zinc fingers were also cloned into pSG5 to form the PLZF 5′ construct. The construct encoding the mutant RARα without the A domain (ΔRARα3′) and pSG5-PML-RARα L were kindly provided

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Abbreviations: APL, acute promyelocytic leukemia; ORF, open reading frame; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RARE, RA response element.

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by H. de The (10). The normal RARα and retinoid X receptor α (RXRα) expression constructs were gifts from R. M. Evans (Salk Institute, San Diego). The expression vectors were cotransfected with luciferase reporter plasmids based on promoters containing RA response elements (RAREs) (10): RARE3-tk-luc, which contains three copies of the RARE in the promoter region of *RARB*, fused to the thymidine kinase gene (*tk*) promoter–luciferase gene (25), and RARβ-pr-luc, containing the native *RARB* promoter region (−5 kb to nucleotide +155) fused to the promoterless luciferase gene (26). Other plasmids used were the *tk* promoter–luciferase construct without any RARE, as negative control; CMV-luc and RSV-luc with the luciferase gene under control of the cytomegalovirus and Rous sarcoma virus promoters, respectively, as positive controls; and the β-galactosidase expression vector (pCH110, Pharmacia) as internal control for each transfection experiment.

Transient Cotransfection. The culture conditions for COS-1 monkey kidney cells, HL-60 human promyelocytic leukemia cells, and U-937 human monocytic cells were as described (10, 27). For COS-1 cells, the calcium phosphate coprecipitation protocol was used (28), whereas a variation of the electroporation techniques of Kakizuka *et al.* (9) was carried out with HL-60 and U-937 cells. In brief, 5×10^6 HL-60 or U-937 cells in 200 μl of opti-MEM (GIBCO) were electroporated (250 V, 960 μF). After transfection, the cells were allowed to recover overnight in RPMI 1640 with 10% fetal bovine serum and then incubated 10–20 hr in the presence of all-*trans*-RA. The transfected DNA contained the reporter plasmid, an expression vector for receptor (RARα, ΔRARα3′, PLZF(A)–RARα, PLZF(B)–RARα, PML–RARa, or PLZF 5′), and 4 μg of β-galactosidase expression vector. Quantities of reporter and expression vectors are specified for each experiment (see *Results* and legends to Figs. 2–4). When necessary, DNA quantity was adjusted with a DNA carrier (pTZ18, Pharmacia). Luciferase activity was measured (29) in a luminometer and luciferase activity is expressed in arbitrary units. Each point is the mean or representative data of at least three independent experiments; standard deviation of the β-galactosidase activity ranged from 10% to 20%.

RESULTS

Molecular Cloning of the Entire *PLZF–RARA* and *RARA–PLZF* ORFs. Previously, we demonstrated by reverse transcription–PCR that in cells carrying the t(11;17) translocation, both PLZF–RARα and RARα–PLZF hybrids were expressed (20). In the present work, the entire ORFs of the reciprocal fusion genes have been cloned, and the fusion proteins have been expressed to investigate their transactivating activities. Sequence analysis of the *PLZF–RARA* fusion transcripts shows that there are two isoforms which are identical but for one putative exon of the *PLZF* gene (bracketed sequence in Fig. 1*A*, designated as exon B). The two isoforms are designated *PLZF(A)–RARA* and *PLZF(B)–RARA*, respectively, according to the absence or presence of exon B. We have also cloned the ORF encoding RARα1–PLZF (Fig. 1*B*). Sequence analysis shows that in the two PLZF–RARα isoforms, the amino-terminal region as well as the two first zinc fingers from PLZF are fused with the B–F domains of RARα. In RARα1–PLZF, the A region of RARα is joined to the carboxyl-terminal region of PLZF, consisting of seven zinc fingers. The PLZF(A)–RARα protein expressed in COS cells has been analyzed by Western blot with a polyclonal antibody directed against the F domain of the human RARα (data not shown). The molecular mass was approximately 95 kDa, similar to that calculated from the predicted amino acid sequence of PLZF(A)–RARα (735 amino acids).

A PLZF-RARα

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MDLTKMGMIQLQNPSHPTGLLCKANQMRLAGTLCDVVIMVDSQEFHAHRT  50
VLACTSKMFEILFHRNSQHYTLDFLSPKTFQQILEYAYTATLQAKAEDLD 100
DLLYAAEILEIEYLEEQCLKMLETIQASDDNDTEATMADGGAEEEEDRKA 150
RYLKNIFISKHSSEESGYASVAGQSLPGPMVDQSPSVSTSFGLSAMSPTK 200
AAVDSLMTIGQSLLQGTLQPPAGPEEPTLAGGGRHPGVAEVKTEMMQVDE 250

VPSQ[QSPGAAESSISGGMGDKVEERGKEGPGTPTRSSVITSARELHYGRE 300

ESAEQVPPPAEAGQAPTGRPEHPAPPPEKHLGIYSVLPNHKADAVLSMPS 350

SVTSGLHVQPALAVSMDFSTYGGLLPQ]GFIQRELFSKLGELAVGMKSESR 400

TIGEQCSVCGVELPDNEAVEQHRKLHSGMKTYGCELCGKRFLDSLRLRMH 450
                ▼
LLAHSAIETQSSSSEEIVPSPPSPPPLPRIYKPCFVCQDKSSGYHYGVSA 500
CEGCKGFFRRSIQKNMVYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGMS 550
KESVRNDRNKKKKEVPKPECSESYTLTPEVGELIEKVRKAHQETFPALCQ 600
LGKYTTNNSSEQRVSLDIDLWDKFSELSTKCIIKTVEFAKQLPGFTTLTI 650
ADQITLLKAACLDILILRICTRYTPEQDTMTFSDGLTLNRTQMHNAGFGP 700
LTDLVFAFANQLLPLEMDDAETGLLSAICLICGDRQDLEQPDRVDMLQEP 750
LLEALKVYVRKRRPSRPHMFPKMLMKITDLRSISAKGAERVITLKMEIPG 800
SMPPLIQEMLENSEGLDTLSGQPGGGGRDGGGLAPPPGSCSPSLSPSSNR 850
SSPATHSP                                            858
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B RARα1-PLZF

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MASNSSSCPTPGGGHLNGYPVPPYAFFFPPMLGGLSPPGALTTLQHQLPV  50
                ▼
SGYSTPSPATGAKAFVCDQCGAQFSKEDALETHRQTHTGTDMAVFCLLCG 100
KRFQAQSALQQHMEVHAGVRSYICSECNRTFPSHTALKRHLRSHTGDHPY 150
ECEFCGSCFRDESTLKSHKRIHTGEKPYECNGCDKKFSLKHQLETHYRVH 200
TGEKPFECKLCHQRSRDYSAMIKHLRTHNGASPYQCTICTEYCPSLSSMQ 250
KHMKGHKPEEIPPDWRIEKTYLYLCYV                         277
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FIG. 1. PLZF–RARα and RARα–PLZF fusion proteins. Amino acid sequences of PLZF–RARα (*A*) and RARα1–PLZF (*B*) are deduced from the cDNA sequences. One-letter codes for amino acids are used. The bracketed sequence (in italics) in PLZF–RARα is encoded by the B exon. The first two PLZF zinc fingers contained in PLZF–RARα and the last zinc fingers contained in RARα1–PLZF are underlined. Filled triangles indicate the junction between PLZF and RARα.

PLZF–RARα Inhibits the Transactivation Properties of Wild-Type RARα in the Presence of All-*trans*-RA. Transcriptional activation mediated by PLZF–RARα in the presence of all-*trans*-RA was studied in COS cells and RA-sensitive human myeloid HL-60 and U-937 leukemic cells. Transfections with the RA-responsive reporter genes (RARβ-pr-luc and RARE3-tk-luc) produced notable levels of luciferase activity (Fig. 2 *A* and *B*). The activation was higher in HL-60 and U-937 cells, probably due to the presence of highly expressed endogenous receptors—namely, RARα and RXRα (ref. 6 and unpublished results). The effect is dose-dependent in HL-60 and U-937 cells, with a dose of 10 nM all-*trans*-RA being sufficient to give a significant induction.

Introduction of vector expressing wild-type RARα further increased the luciferase activity in COS-1 cells and RA-sensitive myeloid cells (Fig. 2 *C* and *D*). However, neither PLZF(A)–RARα nor PLZF(B)–RARα had such an effect. When the RARβ-pr-luc reporter was used, both fusion receptor isoforms tended to decrease the luciferase activity produced by endogenous receptors. In contrast, the RARα mutant lacking the A domain (ΔRARα 3′) could still raise luciferase activity from the RARE reporter in either cell type (Fig. 2 *C* and *D*). As expected, the PLZF 5′ mutant did not affect the RA-mediated endogenous response. This indicates that both PLZF and RARα sequences are necessary for the altered transactivating activity. In the same transfection experiments, the PML–RARα long (L) isoform (10) displayed a transactivating property similar to that observed with PLZF–RARα isoforms (Fig. 2 *C* and *D*).

To further characterize the biological activities of PLZF–RARα compared with the normal RARα, an all-*trans*-RA dose–response analysis was carried out. In the presence of PLZF–RARα, the activation of the reporter gene was not

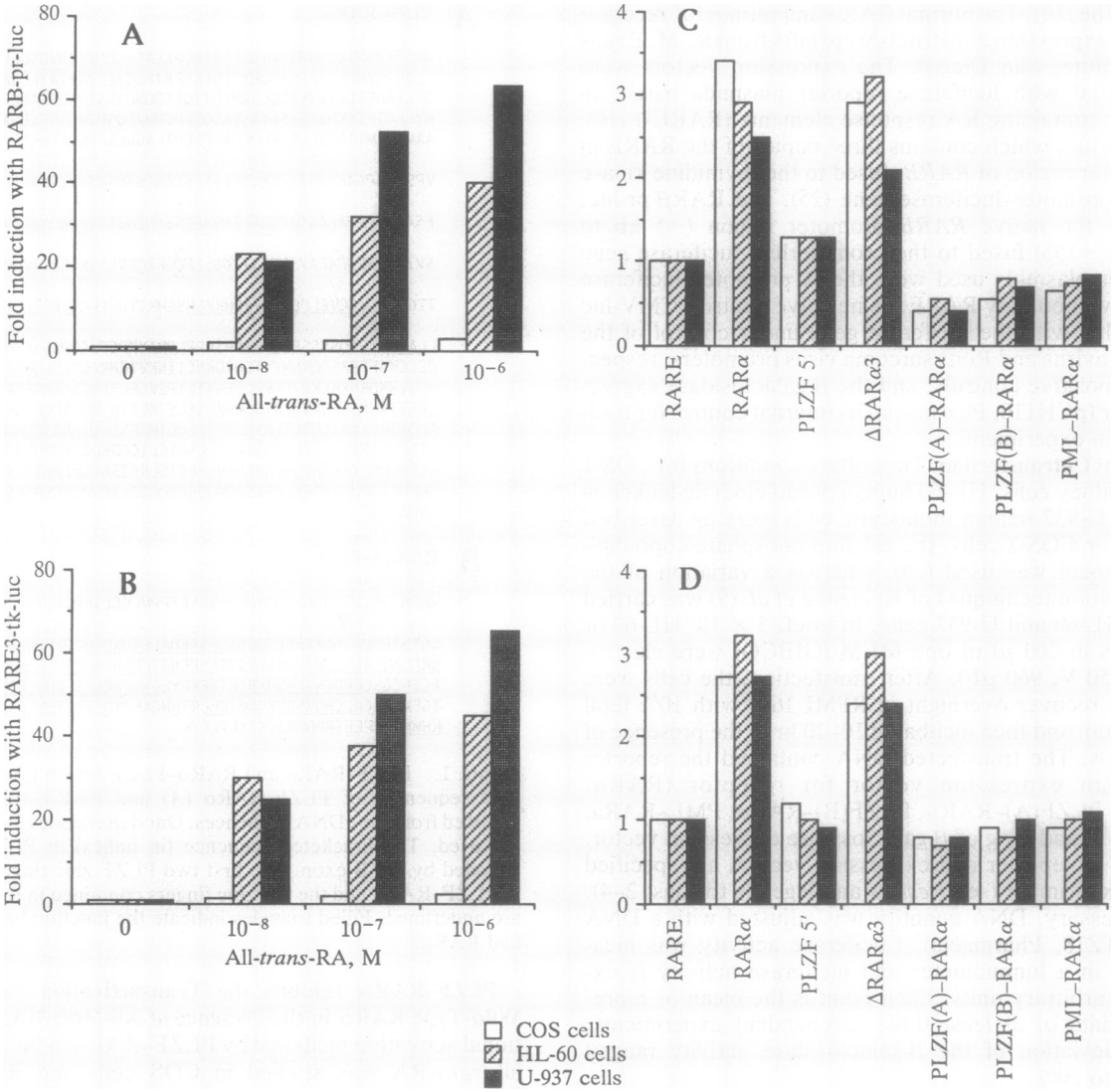

FIG. 2. Transactivation of RA-responsive reporter genes by the PLZF–RARα fusion proteins and by endogenous receptors and transfected RARα. (*A* and *B*) Transactivation properties of endogenous RARs on RARβ-pr-luc (*A*) and RARE3-tk-luc (*B*) reporters in the presence of various concentrations of all-*trans*-RA. Results are expressed as the ratios between the luciferase activities induced by endogenous receptors through each reporter in the presence and absence of all-*trans*-RA. (*C* and *D*) Transactivation of RARβ-pr-luc (*C*) and RARE3-tk-luc (*D*) reporters mediated by the PLZF–RARα fusions, the endogenous receptors (RARE), the wild-type RARα, the truncated PLZF (PLZF 5'), the truncated RARα (ΔRARα3), and PML–RARα in the presence of 1 μM all-*trans*-RA. Results are expressed as the ratio between the luciferase activities induced by transfected receptors and that induced by the endogenous receptors. Tests were carried out in COS-1 (open bars), HL-60 (hatched bars), and U-937 (filled bars) cells. Quantity of each reporter plasmid transfected was 10 μg; that of each test receptor expression vector (*C* and *D*) was 2 μg.

observed even with high concentrations of RA (Fig. 3*A*). These data suggested that PLZF–RARα might antagonize the normal RARα. To confirm this hypothesis, cotransfections of wild-type *RARA* and *PLZF–RARA* together with either reporter (RARE3-tk-luc or RARβ-pr-luc) and at two all-*trans*-RA concentrations (0.1 and 1 μM) were conducted in COS-1, HL-60, and U-937 cells. When the quantity of the transfected *PLZF–RARA* constructs was varied in the presence of a constant amount of *RARA* construct, a significant repression of RARα transactivation activity was maintained even with a much lower *PLZF–RARA* plasmid concentration (representative results in HL-60 cells are shown in Fig. 3*B*). In contrast, increasing the quantity of *RARA* construct in the presence of a constant amount of *PLZF–RARA* construct could not abrogate the inhibitory effect from the latter (representative results in HL-60 cells are shown in Fig. 3*C*).

PLZF–RARα Antagonizes the RARα/RXRα Heterodimer. The RAR/RXR heterodimer is the most active form of the receptor (30, 31). To look at the possible effect of PLZF–RARα on the heterodimer, we performed cotransfection assays using RARα and/or RXRα in the presence of the two active forms of RA, all-*trans*-RA and 9-*cis*-RA. A "dominant negative" effect against RARα and RXRα was present when either of the two fusion receptor isoforms was cotransfected (Fig. 4). This inhibitory effect was not affected by the type of RA used.

DISCUSSION

The t(11;17)(q23;q21) translocation, a rare but recurrent chromosomal event in APL, fuses *RARA* to the gene encoding putative zinc finger transcription factor, *PLZF* (20, 21). Compared with the wild-type RARα, the PLZF–RARα fusion retains the B–F domains of the nuclear receptor but lacks the A domain, which is replaced by the portion encoded by the *PLZF* 5' region. Investigation of the functional properties of this fusion receptor protein with respect to the PML–RARα produced from the t(15;17) translocation and the wild-type

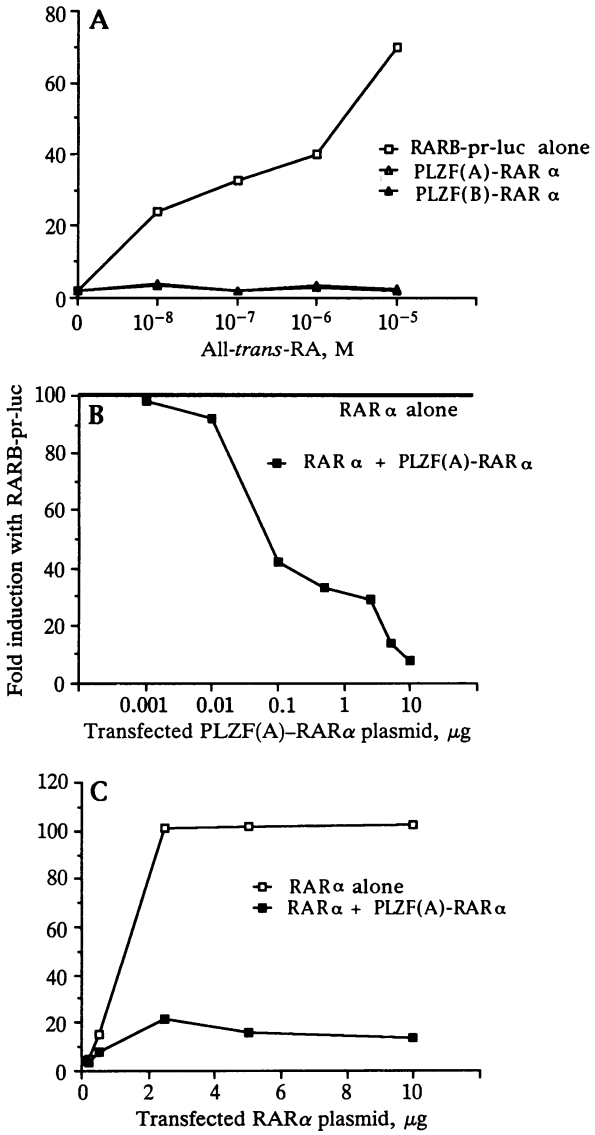

FIG. 3. Effect of various concentrations of ligand and normal or PLZF–RARα fusion receptor expression vectors on the transcription of the RARβ-pr-luc reporter gene in HL-60 cells. (*A*) Dose–response analysis for all-*trans*-RA (0.01–10 μM) in the presence of reporter gene (RARB-pr-luc) alone or in combination with PLZF(A)–RARα (2 μg) or PLZF(B)–RARα (2 μg) expression vector. (*B*) RARβ-pr-luc reporter gene expression in HL-60 cells cotransfected with PLZF(A)–RARα expression vector (0.001–10 μg) and RARα expression vector (2 μg) in the presence of 0.1 μM all-*trans*-RA. Results are expressed as percentage of induction obtained with RARα alone. (*C*) RARβ-pr-luc reporter gene expression in HL-60 cells cotransfected with wild-type RARα expression vector (0.001–10 μg) and a constant quantity of PLZF(A)–RARα expression vector (2 μg) in the presence of 0.1 μM all-*trans*-RA. In all these experiments the RARβ-pr-luc reporter vector was transfected at 10 μg.

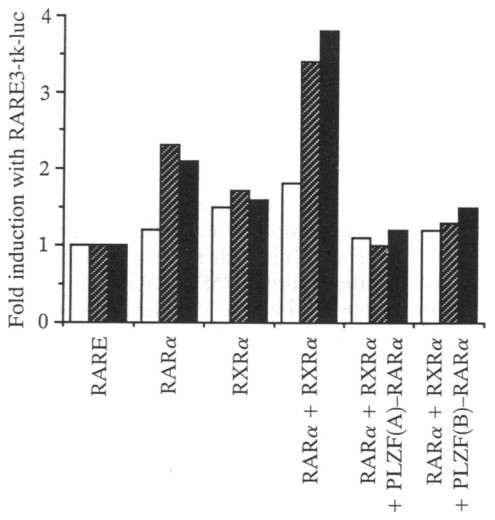

FIG. 4. Transactivation properties mediated by the cotransfection vectors encoding RARα–RXRα and the two PLZF–RARα fusion proteins. PLZF(A)–RARα and PLZF(B)–RARα exhibit a dominant negative effect against RARα/RXRα in the presence of 1 μM all-*trans*-RA (hatched bars) or 9-*cis*-RA (filled bars) or in the absence of RA (open bars). RARα, RXRα, PLZF(A)–RARα, and PLZF(B)–RARα expression vectors were transfected at 1 μg. The experiment was performed in COS-1 cells with RARE3-tk-luc reporter (3 μg). Results are expressed as the ratios of the luciferase activities induced by transfected receptors to that mediated by the endogenous receptor activities.

RARα is of importance for understanding the pathogenesis of APL.

Recently, two groups of receptors, RARs and RXRs, have been shown to mediate the action of various RAs in embryogenesis, differentiation, and cell growth (30, 31), by modulating the transcription of target genes through specific RAREs. Whereas the three RA isomers 13-*cis*-RA, all-*trans*-RA, and 9-*cis*-RA have been identified as the ligands for RARs, the RXRs bind only the 9-*cis* isomer with high affinity. Though these receptors may function as homodimers, RAR/RXR heterodimers bind more efficiently to RAREs. Hence, an abnormal RARα can be predicted to antagonize its wild-type counterpart by competing for binding to RXRα, thus interfering with the normal RARα/RXRα-mediated regulation of myeloid differentiation, and/or acquire new biological properties resulting from the fused sequence.

Using transient cotransfection assays to test this hypothesis, we confirmed in RA-sensitive myeloid cells, through the use of two different reporter plasmids, that the RARα mutant lacking the A domain has no obviously altered transactivating properties. In contrast, the chimeric receptors produced by the t(11;17) translocation, PLZF(A)–RARα and PLZF(B)–RARα, behave differently than the wild-type RARα in COS-1 monkey cells and the human myeloid cell lines HL-60 and U-937, indicating that the functional alteration is due to the fusion to PLZF sequence. Specifically, these PLZF–RARα fusion proteins provoke a dominant negative effect on the transactivation mediated by the endogenous receptor activities and by the transfected wild-type RARα over a large range of all-*trans*-RA concentrations. Moreover, the inhibitory effect persists when both wild-type RARα and RXRα are cotransfected in myeloid cells in the presence of their respective ligands (all-*trans*-RA and 9-*cis*-RA). It is thus possible that the PLZF–RARα fusion proteins function as inhibitors of the transcription activity mediated by the RARα/RXRα heterodimer. In all experiments, PLZF(A)–RARα and PLZF(B)–RARα exhibited the same suppressor effect, suggesting that the presence or absence of the B-form exon is not a crucial factor in the functional properties of the fusion proteins. In view of the wide physiological functions and pathological processes involving RAs and their receptors, the dominant negative effect of the PLZF–RARα fusion proteins observed in the present work should not be generalized to other tissues. However, our study demonstrates that in the myeloid system, the PLZF–RARα fusion proteins abrogate the function of their wild-type counterpart.

Although the partner genes of *RARA* in both classical and variant translocations in APL—namely, *PML* and *PLZF*—encode putative transcription factors, they differ by their structure and differential expression in tissues. The two genes are of different phylogenic origins: *PLZF* belongs to

the Kruppel family (20, 21) whereas *PML* is a member of a newly recognized class encoding zinc finger proteins, including the potential transcription factors Rpt-1, Rfp, and Mel-18 (9–11, 13). The expression pattern is also different because *PML* is ubiquitously expressed (11), whereas *PLZF* expression is more restricted, detected mainly in the hematopoietic system (20). That *PLZF* expression is down-regulated during RA-induced granulocytic maturation suggests that it may play a role in the regulation of myeloid differentiation (20). However, with the myeloid cell system and reporter genes that we have studied, both PML–RARα and the PLZF–RARα fusion proteins behave similarly in terms of the inhibition of RARα transcriptional activities in the presence of RA.

In t(15;17) leukemias, all-*trans*-RA has been shown to abrogate the maturation block and, through differentiation induction, achieve clinical remission in >95% of *de novo* cases (17–19). There is a very good correlation between the *in vitro* and *in vivo* differentiation data in the newly treated cases (17–19). However, preliminary *in vitro* data on a group of APL patients expressing PLZF–RARα predict a poor response to all-*trans*-RA therapy *in vivo* (unpublished data). These different clinical responses to all-*trans*-RA suggest that PML–RARα and PLZF–RARα contribute to the APL leukemogenesis in a similar way but that their biological properties with respect to the APL cell differentiation induced by all-*trans*-RA may be different.

That RARα is the common target in both t(15;17) and t(11;17) suggests its crucial role in the control of myeloid differentiation. The identification of the functional characteristics of PLZF–RARα compared with those of PML–RARα will help to clarify the role played by the altered RARα, PML, PLZF, and corresponding fusion proteins in leukemogenesis and RA sensitivity of APL cells.

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