Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3624–3629, April 1996
Genetics

Amino-terminal protein–protein interaction motif (POZ-domain) is responsible for activities of the promyelocytic leukemia zinc finger–retinoic acid receptor-α fusion protein

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Communicated by Tan Jiazhen, Fudan University, Shanghai, China, November 7, 1995 (received for review September 15, 1995)

ABSTRACT **Promyelocytic leukemia zinc finger–retinoic acid receptor α (PLZF–RARα), a fusion receptor generated as a result of a variant t(11;17) chromosomal translocation that occurs in a small subset of acute promyelocytic leukemia (APL) patients, has been shown to display a dominant-negative effect against the wild-type RARα/retinoid X receptor α (RXRα). We now show that its N-terminal region (called the POZ-domain), which mediates protein–protein interaction as well as specific nuclear localization of the wild-type PLZF and chimeric PLZF–RARα proteins, is primarily responsible for this activity. To further investigate the mechanisms of PLZF–RARα action, we have also studied its ligand–receptor, protein–protein, and protein–DNA interaction properties and compared them with those of the promyelocytic leukemia gene (PML)–RARα, which is expressed in the majority of APLs as a result of t(15;17) translocation. PLZF–RARα and PML–RARα have essentially the same ligand-binding affinities and can bind *in vitro* to retinoic acid response elements (RAREs) as homodimers or heterodimers with RXRα. PLZF–RARα homodimerization and heterodimerization with RXRα were primarily mediated by the POZ-domain and RARα sequence, respectively. Despite having identical RARα sequences, PLZF–RARα and PML–RARα homodimers recognized with different affinities distinct RAREs. Furthermore, PLZF–RARα could heterodimerize *in vitro* with the wild-type PLZF, suggesting that it may play a role in leukemogenesis by antagonizing actions of not only the retinoid receptors but also the wild-type PLZF and possibly other POZ-domain-containing regulators. These different protein–protein interactions and the target gene specificities of PLZF–RARα and PML–RARα may underlie, at least in part, the apparent resistance of APL with t(11;17) to differentiation effects of all-*trans*-retinoic acid.**

Nonrandom chromosomal translocations play an important role in the pathogenesis of human malignant hemopathies (1, 2). Three different chromosomal translocations have so far been reported and characterized in acute promyelocytic leukemia (APL) (3–5). In the great majority of patients, there is a specific chromosomal translocation t(15;17)(q22;q21), which involves the promyelocytic leukemia (*PML*) gene located on chromosome 15 and the retinoic acid receptor α (*RARα*) gene located on chromosome 17 (3, 6–11). Almost all t(15;17) APL patients respond well to the differentiation therapy with all-*trans*-retinoic acid (ATRA) (12). In a small subset of APLs a second translocation t(11;17)(q23;q21) has been found that fuses the RARα locus with the promyelocytic leukemia zinc finger (*PLZF*) gene on chromosome 11q23 (4, 13, 14). In contrast to patients with t(15;17) (12), the APL patients with t(11;17) have poor response to ATRA (15). Recently, an APL patient has been described who had yet another variant translocation, t(5;17), involving the RARα locus and a known gene on chromosome 5 called *NPM* (nucleophosmin) (5). The *NPM* gene, which encodes a nucleolar phosphoprotein possibly involved in ribosome processing or assembly, has also been implicated in the t(2;5) translocation in anaplastic lymphomas, which results in expression of an NPM–ALK fusion gene product (2, 16).

The fact that in three translocations RARα is the common target strongly suggests that the disturbance of retinoid signaling and RARα/retinoid X receptor α (RXRα) function in mediating cellular differentiation plays a key role in APL pathogenesis. However, the difference in clinical response to ATRA treatment between t(15;17) APL patients and those with t(11;17) could be due to functional differences of the fusion genes resulting from the two translocations. In this study, we have addressed the structural and functional aspects of *PLZF–RARα* fusion gene product in order to better understand the molecular basis for the potentially altered activities of the chimeric protein. The ligand–receptor, protein–protein, and protein–DNA interactions of PLZF–RARα were studied and compared, when possible, to those of PML–RARα.

MATERIALS AND METHODS

Plasmid. The *RARα*, *PLZF*, *PLZF–RARα* expression vectors and reporter plasmid RARβ-pr-luc were as described (17). The human *RXRα* (in pCMX vector) plasmid was a gift from R. M. Evans (Salk Institute, San Diego) (18). The *PML–RARα* (in pSG5 vector) expression plasmid used in these experiments was previously constructed in our laboratory. Following *PLZF–RARα* expression vectors, with deletions of different PLZF motifs, were generated (Fig. 1*A*): C.I, deletion of amino acids 172–348 containing 4 proline-dependent phosphorylation sites; C.II, deletion of amino acids 403–432 corresponding to the first zinc finger structure of PLZF; C.III, deletion of amino acids 432–455, thus lacking the second zinc

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Abbreviations: APL, acute promyelocytic leukemia; PLZF, promyelocytic leukemia zinc finger; PML, promyelocytic leukemia gene; POZ, poxvirus and zinc finger; RARα, retinoic acid receptor α; RXRα, retinoid X receptor α; ATRA, all-*trans*-RA; NPM, nucleophosmin; PML, promyelocytic leukemia; RARE, RA response element; DR, direct repeat; GST, glutathione *S*-transferase.

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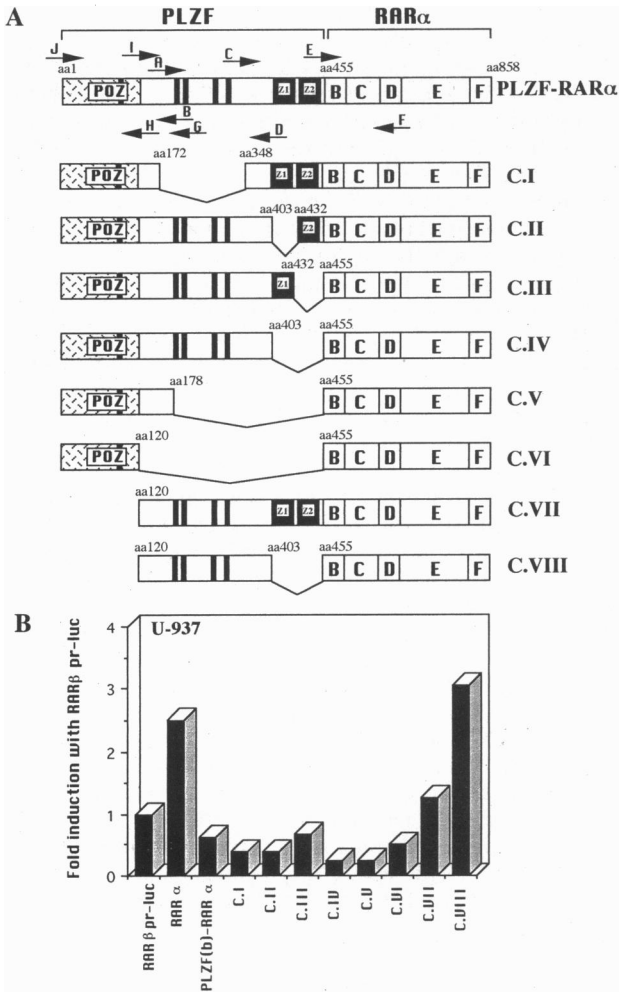

FIG. 1. (*A*) Schematic illustration of PLZF–RARα constructs with deletion of different motifs of the PLZF portion (see text). Arrows A, B, C, D, E, F, G, H, I, and J show positions of primers used to construct these PLZF–RARα constructs. Five solid vertical bars represent potential $x^s/_T Px$ proline-dependent phosphorylation targets. Z1 and Z2 show the first and second zinc finger of PLZF, respectively, and B, C, D, E, and F show the B, C, D, E, and F domains of RARα. (*B*) Transactivation activities of PLZF–RARα lacking different functional domains of PLZF compared to wild-type RARα and native PLZF–RARα in U-937 cells. The ATRA concentration of cell culture medium was 10^{-6} M. Similar results were obtained in three different experiments. Experiments with lower ATRA concentrations gave concordant results with a lower luciferase expression level. Leftmost column represents the single transfection with reporter plasmid, while all other columns represent results of cotransfection of both RA receptor expression vectors and reporter plasmid.

finger region; C.IV, amino acids 403–455 containing the first and second zinc finger motifs are deleted; C.V, deletion of amino acids 178–455; C.VI, deletion of amino acids 120–455, thus retaining only the POZ-domain; C.VII, deletion of amino acids 1–120; C.VIII, deletion of amino acids 1–120 and 403–455 corresponding to the POZ-domain and the two zinc finger motifs, respectively. The *PLZF* (*−POZ*) with deletion of the POZ-domain was also generated. These plasmids have been checked by DNA sequencing and by *in vitro* translation assay.

***In Vitro* Translation.** One microgram of plasmid was added to TNT-coupled rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. To estimate the relative quantity of *in vitro* translated proteins, parallel translation reactions were performed in the presence of [^{35}S]methionine (1200 Ci/mmol; 1 Ci = 37 GBq) (NEN) and the proteins were visualized by autoradiography after separation on a SDS/8% polyacrylamide gel. The quantity of proteins used in ligand binding and gel-shift assays was normalized according to the ^{35}S incorporation rate and the same amount of proteins was used in the comparative study of different receptors.

Ligand Binding Assays of RARα, PML–RARα, and PLZF–RARα. *In vitro* translated RARs were incubated with different concentration of [^{3}H]RA (0.1–10 nM) (Sigma) for 20 hr at 4°C. Nonspecific binding was measured in the presence of 100- to 200-fold excess of unlabeled RA. To each incubated sample PTG buffer (5 mM sodium phosphate, pH 7.4/10 mM thioglycerol/10% glycerol/2 units of aprotinin per ml/2 units of leupeptin per ml/1 mM phenylmethylsulfonyl fluoride) was added to a final vol of 0.8 ml. Bound radioactivity was separated from the free fraction by eluting samples from PD-10 desalting columns (Pharmacia) with 5 mM phosphate buffer, pH 7.4/400 mM KCl/10% glycerol/1 mM dithiothreitol (DTT)/1 mM phenylmethylsulfonyl fluoride. Bound radioactivity was eluted in the void volume and was quantified by liquid scintillation counting of the eluent. Saturation curve analysis was performed with a Goldstain model fit saturation curve (19). Each K_d value was the mean of at least two independent experiments.

Transient Cotransfection. The calcium phosphate coprecipitation and electroporation protocols used for transfections of COS-1 (17), U-937 (8), and 293T (20) cells were as described. The transfected DNA contained the reporter plasmid, an expression vector for one of the receptor proteins [RARα, PLZF(b)-RARα, C.I, C.II, C.III, C.IV, C.V, C.VI, C.VII, or C.VIII], and 4 μg of β-galactosidase expression vector as internal control. Luciferase activity was measured in a luminometer, expressed in arbitrary units and normalized according to the internal control (21). Each point is the mean of at least three independent experiments.

Gel-Shift DNA-Binding Assays. The two RA response element (RARE) probes DR5G and DR5T (β_2RARE) (22) were used in this experiment. The sequences of the ^{32}P-labeled RAREs, with the direct repeat (DR) sequences underlined are as follows: DR5G, 5′-GGGTAGGGGTCACCGAAAGGTCACTCG-3′; DR5T (β_2RARE), 5′-GGGTAGGGTTCACCGAAAGTTCACTCG-3′. The *in vitro* translated proteins were preincubated for 15 min at room temperature in the following buffer: 20 mM Hepes, pH 7.4/50 mM KCl/1 mM 2-mercaptoethanol/10% glycerol/1 μg of poly(dI-dC) (Pharmacia)/100 μg of bovine serum albumin (BSA). ^{32}P-labeled probe (60,000–80,000 cpm per assay corresponding to ≈10 fmol of probe) was then added and the samples were further incubated at room temperature for 30 min and at 4°C for 30 min (total vol, 20 μl). Protein–DNA complexes were resolved on 6% polyacrylamide gels equilibrated in 0.5% TBE and run at 250 V. The gels were fixed in 10% acetic acid and 30% methanol for 15 min, dried, and autoradiographed at −80°C.

Immunofluorescence. For immunofluorescence, cytospins of 293T cells, transfected with appropriate expression vectors, were prepared and cells were fixed for 20 min at room temperature in 4% paraformaldehyde. Subsequently, cells were washed several times in Ca^{2+}- and Mg^{2+}-free PBS and then incubated for 10 min in 0.2% Triton X-100. After an additional wash in PBS, rabbit anti-human PLZF antiserum (23) diluted 1:200 in PBS was applied for 2 hr. After several 10-min washes in PBS, donkey anti-rabbit fluorescein isothiocyanate-conjugated immunoglobulin (Jackson ImmunoResearch) was applied for 1 hr. After final washes, the slides were mounted and stained cells were visualized and photographed using a Bio-Rad MRC600 confocal imaging system.

Glutathione *S*-Transferase (GST) "Pulldown" Experiments. Human PLZF protein was expressed in bacteria as a GST fusion product and purified by standard methodology (24). pGST-PLZF plasmid was constructed by inserting the 2.1-kb PLZF cDNA (4) into the *Eco*RI site of pGEX3X vector (Pharmacia). A modified GST pulldown procedure was carried

out as described (25) using 1 μg of GST or GST–PLZF protein on glutathione-Sepharose beads and 3 μl of a given *in vitro* translation product made using TNT-coupled reticulocyte lysate (Promega). Incubation was carried out for 1 hr in Z buffer (25 mM Hepes, pH 7.5/12.5 mM $MgCl_2$/20% glycerol/0.1% Nonidet P-40/150 mM KCl/200 mM DTT/5 mg of BSA/200 μg of EtBr per ml). Washes before and after incubation were carried out in NET-80 (20 mM Tris·HCl, pH 8.0/80 mM NaCl/1 mM EDTA) and NETN buffer (20 mM Tris·HCl, pH 8.0/80 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/200 μg of EtBr per ml), respectively. After the final centrifugation at 500 × *g* for 30 sec, bound proteins were resolved on SDS/polyacrylamide gels.

Immunoprecipitation Assays. For immunoprecipitation assays, 5 μl of *in vitro* translated PLZF protein fused to the "flag" epitope and 5 μl of *in vitro* translated C.VII, CVIII, or PLZF–RARα protein labeled with [^{35}S]methionine was diluted in IP buffer (100 mM KCl/20 mM Hepes, pH 7.4/1 mg of BSA per ml/0.1% Nonidet P-40/10% glycerol) to a final vol of 50 μl and incubated at 4°C overnight with 2 μg of anti-flag M2 antibody (Kodak). Then, 50 μl of a 50% slurry of protein A-Sepharose (Pharmacia) was added and, after incubation at 4°C for 2 hr with rotation, the beads were recovered by centrifugation and washed three times with IP buffer. The proteins were eluted by boiling beads in SDS gel loading buffer and analyzed on SDS/8% polyacrylamide gels.

RESULTS

PLZF–RARα Displays Ligand-Binding Affinity Similar to PML–RARα and Wild-Type RARα. The mean K_d values for ligand binding by the wild-type RARα, PLZF–RARα, and PML–RARα were 1.78, 1.63, and 2.24 nM, respectively. It is worth noting that the K_d values obtained here for RARα and PML–RARα are about an order of magnitude higher than those previously reported using nuclear extracts of cells transfected with the RARα and PML–RARα expression vectors (26), probably reflecting the difference in the methods used. Nevertheless, in this assay both PLZF–RARα and PML–RARα show ligand-binding affinities similar to that of wild-type RARα.

POZ-Domain Is Chiefly Responsible for the Dominant-Negative Properties of the PLZF–RARα Proteins. We have previously shown that PLZF–RARα exerts a dominant-negative effect against the wild-type RARα. This effect seemed to be mediated by the PLZF moiety in the fusion receptor (17), which includes from the N terminus, the POZ (for poxvirus and zinc finger, see below) domain (27), a negatively charged α-helical region, a proline-rich region and two C_2-H_2 zinc finger motifs. It was shown that the proline-rich domain did not contribute to the dominant-negative effect (17). Nevertheless, which of the other element(s) of PLZF may be responsible for this effect was not determined. To study the structure–function relationship of these domains, we constructed PLZF–RARα mutants with deletions of different PLZF motifs. When the RA-responsive reporter plasmid (RARβ-pr-luc) was transfected into U-937 cells, notable levels of luciferase activity were produced due to the activities of the endogenous RARs/RXRs (Fig. 1*B*). Cotransfection of the wild-type *RARα* further increased the level of the reporter gene expression. In contrast, cotransfection of *PLZF–RARα* vector (or *PLZF–RARα* and *RARα*) decreased the luciferase activity produced by this reporter plasmid in U-937 (Fig. 1*B*) cells (see also ref. 17). The expression vectors C.I, C.II, C.III, and C.IV had similar effects, indicating that the negatively charged region with potential proline-dependent phosphorylation sites and the two zinc finger domains alone are not critical for maintenance of the dominant-negative effect. Moreover, expression of C.V (retaining only amino acids 1–178, which contain the POZ-domain and a short negatively charged region) and C.VI (lacking all PLZF motifs except for the POZ-domain) could not increase the reporter gene activity. However, expression of C.VIII (lacking the POZ-domain and the two zinc fingers) increased significantly the luciferase activity produced by reporter plasmid in U-937 cells. Transactivation of reporter gene in the presence of C.VII (lacking only the POZ-domain) was lower than when C.VIII was present. Since the POZ-domain was deleted in both C.VII and C.VIII, the above results indicate that it plays a critical role in inhibition of transactivation of the above reporter gene, while the presence of the two zinc fingers appears to enhance this effect. In COS-1 cells, cotransfection of expression vector C.VIII also allowed significant transactivation, confirming results in U-937 cells (data not shown). Taken as a whole, these results demonstrated that the POZ-domain (amino acids 1–120) is crucial for the dominant-negative properties of PLZF–RARα and the two zinc fingers may enhance these activities through an as yet unknown mechanism.

PLZF–RARα Binds to RAREs by Forming a Homodimer or a Heterodimer with RXRα. *In vitro* translated receptor proteins were tested for their RARE-binding abilities by gel-shift assay. As shown in Fig. 2*A*, PLZF–RARα can bind, relatively weakly compared to wild-type RARα–RXRα heterodimer, to DR5G and DR5T in the absence of RXRα. The addition of 100-fold cold cognate RARE oligonucleotide in the same system inhibited the appearance of the shifted band, indicating specificity of these results (Fig. 2*B*). According to the pattern of mobility shift, with RARα/RXRα as size reference, the retarded bands in lanes 3 and 6 of Fig. 2*A* should correspond to the homodimer of PLZF–RARα. In addition, PLZF–RARα can form a heterodimer with RXRα because four retardation bands of distinct sizes were consistently produced on repeated gel-shift assays after coincubation of the two proteins (Fig. 2*C*). These four bands correspond to different

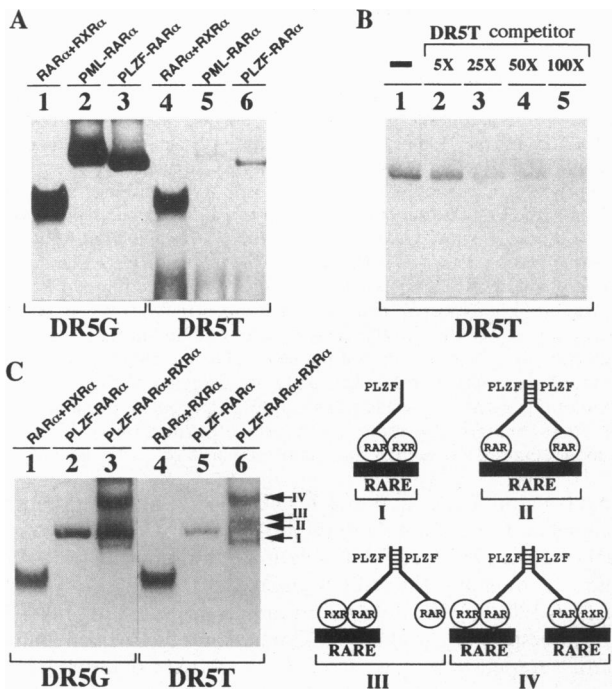

FIG. 2. (*A*) Gel-shift assay of PLZF–RARα RARE (DR5G and DR5T)-binding compared to that of PML–RARα and wild-type RARα–RXRα (lanes 1–6). Note that PLZF–RARα and PML–RARα homodimers bind RARE less efficiently than RARα–RXRα. PLZF–RARα and PML–RARα bind with different affinity to DR5G and DR5T. Similar results were obtained in two independent experiments. (*B*) Specificity test of PLZF–RARα receptor–RARE binding. (*C*) Gel-shift assay of PLZF–RARα with RXRα and schematic description of the four possible PLZF–RARα–RXRα complexes.

complexes, probably representing PLZF–RARα/RXRα heterodimer (I), PLZF–RARα homodimer (II), PLZF–RARα homodimer plus one RXRα (III), and PLZF–RARα homodimer with two RXRα molecules (IV) (Fig. 2*C*). Addition of ATRA or 9C-RA to PLZF–RARα proteins resulted in a more intense band in comparison to the same protein without ATRA or 9C-RA, indicating that both RA isomers can bind to PLZF–RARα and enhance DNA–protein complex formation. In the presence of RXRα, this enhancement of RARE binding appeared stronger (data not shown).

POZ-Domain Mediates Formation of PLZF–RARα Homodimer and PLZF–RARα/PLZF Heterodimer. POZ-domain, an N-terminal motif of ≈120 amino acids whose sequence has been highly conserved through evolution (33), characterizes a subfamily of C_2-H_2 zinc finger proteins. Studying another member of this subfamily, called ZID, Bardwell and Treisman (27) showed that the POZ-domain can mediate protein–protein interaction *in vitro* and determine a specific nuclear localization pattern of the ZID protein. We now show that as in the case of the ZID protein, the POZ-domain of PLZF is required for its self-association *in vitro* and appears to be responsible for specific punctate nuclear localization of PLZF as well as chimeric PLZF–RARα proteins (Figs. 3 and 4). Both PLZF and PLZF–RARα bind to GST–PLZF but not to GST alone *in vitro* (Fig. 3*A*); in contrast, PLZF without the POZ-domain, as well as the RARα protein, did not bind to GST–PLZF *in vitro*, indicating specificity of this association and the requirement of the POZ-domain. It is worth noting that the interaction between PLZF and PLZF–RARα appears stronger than between the two PLZF molecules (see also below), suggesting that in leukemic cells with t(11;17) the

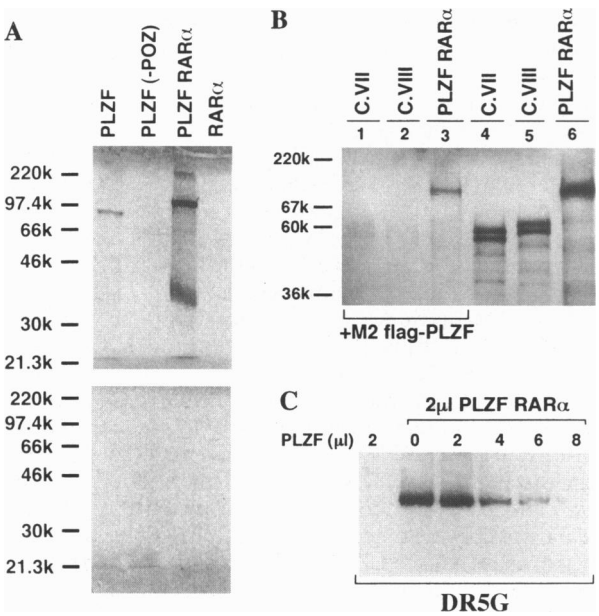

FIG. 3. (*A*) GST–PLZF (*Upper*) or GST (*Lower*) bound to glutathione-Sepharose beads was used to pull-down *in vitro* translated PLZF, PLZF without the POZ-domain [PLZF(−POZ)], PLZF–RARα, and RARα, as indicated above each lane. *In vitro* translated protein was labeled with [^{35}S]methionine. (*B*) Immunoprecipitation assays with *in vitro* translation proteins PLZF–RARα, M2 flag–PLZF, C.VII, and C.VIII lacking the POZ-domain. Lanes 1–3, SDS/PAGE results of C.VII, C.VIII, and PLZF–RARα after immunoprecipitation with anti-flag M2 antibody, respectively; lanes 4–6, *in vitro* translated proteins from C.VII, C.VIII, and PLZF–RARα as a size control. (*C*) When increasing amounts of PLZF protein were preincubated with a constant quantity of PLZF–RARα, the intensity of the shifted bands corresponding to the PLZF–RARα homodimer capable of RARE binding was gradually reduced, indicating that PLZF–RARα forms a heterodimer with PLZF, which is unable to bind RARE.

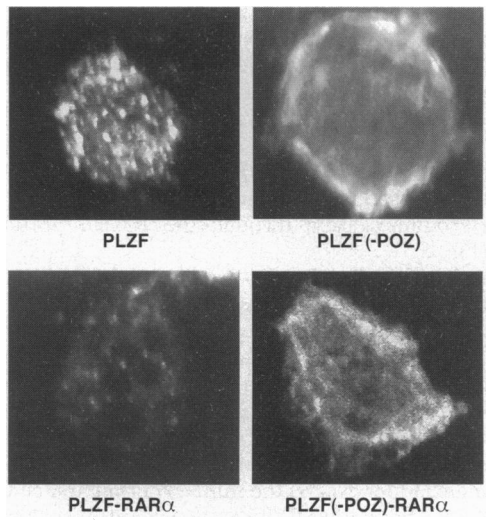

FIG. 4. Nuclear localization patterns of PLZF and PLZF–RARα proteins with and without (−POZ) the POZ-domain as indicated. Only one representative cell is shown from each experiment.

PLZF–RARα:PLZF heterodimer formation is favored over PLZF homodimerization. These data are corroborated by *in vitro* immunoprecipitation experiments (Fig. 3*B*). As shown in Fig. 3*B*, when *in vitro* translated PLZF–RARα and M2 flag–PLZF were subjected to immunoprecipitation with anti-flag antibody, the band corresponding to radiolabeled PLZF–RARα was coprecipitated. Nevertheless, with fusion receptors lacking the POZ-domain (C.VII or C.VIII), the coprecipitation was not seen. The POZ-domain appears to be also required for formation of the PLZF–RARα homodimer, which can bind to RAREs. Using proteins expressed *in vitro* from C.I through C.VI plasmids (Fig. 5), retardation bands corresponding to the expected sizes of the PLZF–RARα homodimer appeared on gel-shift assay. In contrast, no retardation bands were produced using C.VII and C.VIII, which have a deletion of the POZ-domain. Similar results were also

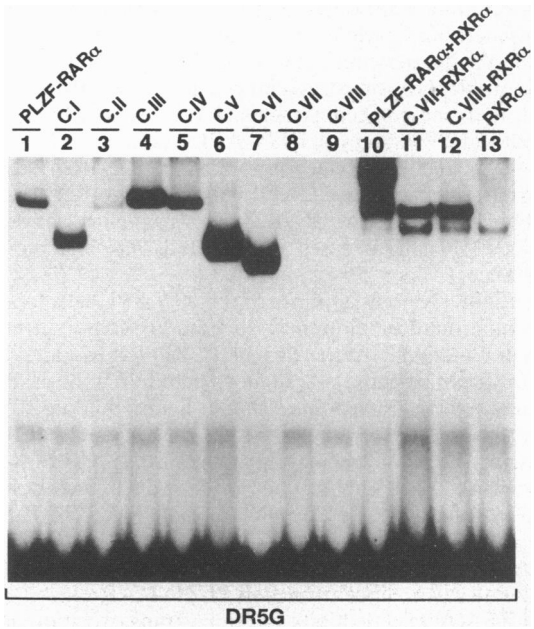

FIG. 5. Gel-shift assay using PLZF–RARα lacking different functional domains of PLZF (lanes 2–9) compared to the PLZF–RARα (lane 1). Note that CVII and VIII (lanes 8 and 9) lacking a POZ motif are unable to bind to RARE. However, CVII/RXRα and CVIII/RXRα (lanes 11 and 12) can bind to RARE.

obtained with proteins expressed after transfection in COS-1 cells (data not shown). The results showing disappearance of the PLZF–RARα homodimer–RARE complex upon addition of increasing amounts of *in vitro* translated PLZF protein further indicated, albeit indirectly, that PLZF–RARα can form a heterodimer with PLZF, which is unable to bind efficiently to the RARE (see Fig. 3*C*). As expected, PLZF–RARα lacking the POZ-domain could still bind to the RARE after heterodimerization through the RARα portion with RXRα (Fig. 5).

Comparison of the RARE-Binding Behavior of PLZF–RARα to That of PML–RARα. It was reported that PML–RARα can bind to RAREs by forming a homodimer or a heterodimer with RXRα and can deregulate the expression of RA-responsive genes (22). In this study, we confirmed a previous report by Perez et al (22) that PML–RARα bound to RAREs less efficiently than RAR–RXR complexes and that its binding to DR5G was stronger than to DR5T (Fig. 2*A*). Interestingly, under exactly the same experimental conditions, PLZF–RARα bound to DR5G with lower affinity than PML–RARα. However, the binding of PLZF–RARα to DR5T is stronger than that of PML–RARα. In addition, when approximately the same amount of chimeric receptors was used in the presence of the same quantity of wild-type RXR, PML–RARα tended to form only two major bands, the homodimer and a complex of homodimer plus one RXR (data not shown); in contrast, the PLZF–RARα/RXR mobility-shift pattern consisted of four bands (Fig. 2*C*).

DISCUSSION

In t(15;17) APLs, the presence of *PML–RARα* in every patient and the absence of reciprocal fusion *RARα–PML* in ≈30% of cases suggests that PML–RARα is crucial in leukemogenesis (28). The wild-type PML is a component of a newly identified nuclear structure of yet unknown function referred to as PML nuclear body or POD (for PML oncogenic domain) (29–32). The POD structures are disrupted in APL cells due to the action of PML–RARα and ATRA treatment restores their integrity while inducing APL cells to differentiate. It is worth noting that wild-type PLZF, which may be involved in a number of important physiological processes including neural development and early hematopoiesis (33), as well as PLZF–RARα, also possess punctate nuclear localization, suggesting that sequestration of coregulators such as RXRα by fusion receptors to these specific nuclear locations could play a role in both t(15;17) and t(11;17) APLs (see also below). A PLZF–RARα chimeric receptor, like PML–RARα, exhibits a strong dominant-negative effect against RARα–RXRα (17). It is still uncertain whether RARα–PLZF, which is expressed in a few t(11;17) APL cases investigated, has any biological importance.

The relatively poor response of t(11;17) APL cells to ATRA differentiation induction may be due to some differences between functional properties of PLZF–RARα and PML–RARα. In this study, we demonstrated that both PLZF–RARα and PML–RARα have similar ligand-binding affinities and both chimeric receptors show the enhancement of RARE binding by RA isomers. Therefore, the insensitivity of t(11;17) APL cells to ATRA cannot be due to a difference in ligand-binding properties of PLZF–RARα and PML–RARα. It is more likely that this insensitivity is related to the unique features of protein–protein and/or protein–DNA interaction of PLZF–RARα. Since truncated PLZF–RARα lacking all of the PLZF sequence exhibits the same transactivation activity as that of wild-type RARα, PLZF sequences should be important in generating the abnormal behavior of PLZF–RARα. Using transient transfection assays, we found that the POZ-domain is mainly responsible for abnormal activities of the PLZF–RARα dimeric protein. The inability of the PLZF–RARα lacking the POZ-domain to form a homodimer, to localize in discrete subnuclear compartments, and to exert a dominant-negative effect against the wild-type RARα–RXRα strongly implies that the POZ-domain of the PLZF–RARα fusion gene may be very important for cellular transformation. It is noteworthy that another gene encoding a POZ-domain zinc finger protein LAZ3/BCL6 (34), is involved in chromosomal translocation t(3;14) in diffuse large cell lymphoma. This represents a second example of a POZ-domain zinc finger protein involvement in oncogenesis, further underlying the potential functional importance of this N-terminal protein–protein interaction motif in mechanisms of cellular transformation.

Like PML–RARα (10, 22), PLZF–RARα can also form a heterodimer or other complexes with RXRα. The interaction between PLZF–RARα and RXRα is mediated by the RARα portion in the fusion receptor. This strong interaction will sequester the RXRα and abrogate the RARα–RXRα as well as other regulatory pathways requiring RXRα. In addition, the fact that PLZF–RARα–RXRα heterodimers can bind to the RARE in the absence of the POZ-domain suggests that the action of the PLZF–RARα homodimer, perhaps on as yet unknown target genes, is also important in the pathogenesis of APL. This may also be true for PML–RARα and, indeed, homodimer formation is an important common denominator between the two RARα fusion proteins. In this respect, it is also worth noting that the NPM portion of the NPM–RARα also possesses a hydrophobic N terminus, which is required for interaction between (NPM) proteins (35).

It appears from our results that homodimerization between PLZF–RARα as well as heterodimerization between PLZF and PLZF–RARα are favored over potentially normal PLZF homodimeric interactions. Therefore, in addition to deregulation of the retinoid signaling pathway, PLZF–RARα can function in leukemogenesis by also antagonizing the function of the wild-type PLZF, perhaps through prevention of its homodimerization. The fact that both PML and PLZF as well as their RARα chimeric proteins have specific nuclear localization patterns opens up the intriguing possibility that despite its different clinical course there may be some common elements between the APLs with t(15;17) and t(11;17) translocations. It remains to be seen, however, if there is any functional relationship between the PML and PLZF proteins and if they colocalize in the nucleus.

In the present work, we also show that the binding of PLZF–RARα to distinct RAREs is not exactly the same as that of PML–RARα. Therefore, PLZF–RARα may have a different target gene specificity than PML–RARα. The different protein–protein and protein–DNA interaction of PLZF–RARα from those of PML–RARα could underlay the basis of distinct response to ATRA by t(11;17) and t(15;17) APL cells *in vitro* and *in vivo*. Although ATRA can restore the localization and possibly the function of PML–RARα, the modulatory effect of ATRA on PLZF–RARα may not be sufficient to relieve the inhibition of both retinoid and PLZF regulatory pathways. To understand well the leukemogenesis and mechanisms underlying the RA differentiation therapy, it will be of great interest to clarify the function of PML and the POZ and to identify the genes whose expression is regulated by the PLZF. Equally important is to isolate genes whose expression is regulated by RA and their receptors (including the wild-type and fusion receptors). Since the replacement of the RARα A region with a sequence containing PML or PLZF dimerization interface essentially stabilizes the formation of a RARα homodimer, which does not normally occur and which appears to have different RARE binding specificities than the RARα–RXRα heterodimer (22), it also remains an important goal to identify genes that are specifically targeted by the RARα chimeric proteins but that may not necessarily be under the control of a RARα–RXRα heterodimer.

We thank Mr. Gang Fu, Ms. Carol Shreiber, and Ms. Ping Zhao for excellent technical assistance and Drs. Hughes de The and Phillip Kastner for constructive discussion. This work is partly supported by the National Natural Science Foundation of China, the Shanghai Municipal Foundation for Natural Sciences, the Samuel Waxman Cancer Research Foundation, the Shanghai "New Star" Research Programme, the Leukaemia Research Fund of Great Britain, the Clyde Wu Foundation of Shanghai Institute of Hematology, and the National Institutes of Health (Grant CA59936 to A.Z., S.W., and J.L.).

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