AML-associated Translocation Products Block Vitamin D₃-induced Differentiation by Sequestering the Vitamin D₃ Receptor

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ABSTRACT

Acute myeloid leukemia (AML)-associated chromosomal translocations result in formation of chimeric transcription factors, such as PML/RAR, PLZF/RARα, and AML-1/ETO, of which the components are involved in regulation of transcription by chromatin modeling through histone acetylation/deacetylation. The leukemic differentiation block is attributed to deregulated transcription caused by these chimeric fusion proteins, which aberrantly recruit histone-deacetylase (HDAC) activity. One essential differentiation pathway blocked by the leukemic fusion proteins is the vitamin (Vit) D₃ signaling. Here we investigated the mechanisms by which the leukemic fusion proteins interfere with VitD₃-induced differentiation. The VitD₃-receptor (VDR) is, like the retinoid receptors RAR, retinoid X receptor, and the thyroid hormone receptor (TR), a ligand-inducible transcription factor. In the absence of ligand, the transcriptional activity of TR and RAR is silenced by recruitment of HDAC activity through binding to corepressors. In the presence of ligand, TR and RAR activate transcription by releasing HDAC activity and by recruiting histone acetyltransferase activity. Here we report that VDR binds corepressors in a ligand-dependent manner and that inhibition of HDAC activity increases VitD₃ sensitivity of HL-60 cells. Nevertheless, the inhibition of HDAC activity is unable to overcome the block of VitD₃-induced differentiation caused by PLZF/RARα expression. Here we demonstrate that the expression of the translocation products PML/RARα and PLZF/RARα impairs the localization of VDR in the nucleus by binding to VDR. Furthermore, the overexpression of VDR in U937 cells expressing AML-related translocation products completely abolishes the block of VitD₃-induced differentiation. Taken together these data indicate that the AML-associated translocation products block differentiation not only by interfering with chromatin-modeling but also by sequestering factors involved in the differentiation signaling pathways, such as VDR in the VitD₃-induced differentiation.

INTRODUCTION

AML is characterized by a block of terminal differentiation of hematopoietic precursors and repression of normal hematopoiesis by the expanding immature blasts. AMLs are associated frequently with specific chromosomal translocations, resulting in the generation of chimeric genes (1, 2). These genes encode chimeric transcription factors, which possess chromatin-modeling activity and deregulate transcription of specific target genes (1, 2).

Ectopic expression of these fusion proteins in several cell models recapitulates the leukemic phenotype by inducing refractoriness to various inducers of myeloid differentiation. Depending on the stimuli, undetermined myeloid precursors are driven toward granulocytic, erythroid, thrombopoietic, or monocytic differentiation. Granulocytic differentiation induced by DMSO, granulocyte colony-stimulating factor, or t-RA (3–7), monocytic differentiation induced by 12-O-tetradecanoylphorbol-13-acetate, granulocyte colony-stimulating factor/granulocyte macrophage colony-stimulating factor, or VitD₃ (4, 6–10), and erythroid differentiation induced by heme or erythropoietin (11), as well as megagarcocyte differentiation induced by phorbol butyrate or thrombopoietin (12) are blocked by the expression of AML-associated translocation products such as PML/RARα, PLZF/RARα, or AML-1/ETO (Fig. 1). Accordingly, the expression of PML/RARα as well as PLZF/RARα in animal models leads to the development of a leukemic phenotype (13–16). In contrast, AML1/ETO-expressing transgenic mice neither exhibit evident alterations of hematopoiesis nor develop leukemia. Nevertheless, hematopoietic progenitor cells expressing AML-1/ETO present impaired differentiation as well as a deregulated proliferation (17).

The mechanisms by which the AML-associated translocation products interfere with the various myeloid differentiation pathways are not completely understood. The fusion proteins PML/RARα, PLZF/RARα, and AML-1/ETO aberrantly recruit HDAC activity, a process considered to be crucial (9, 18). AML-1 is associated with the p300/CBP histone-acetyltransferase and acts as an activator of transcription. In contrast, ETO recruits HDAC by interacting with N-CoR. Thereby, fusion with ETO converts the AML-1 transcriptional activator, which is indispensable for normal hematopoiesis, into a transcriptional repressor (9, 10). Like ETO, PLZF is a transcriptional repressor that strongly binds HDAC and members of the HD-NCR, such as N-CoR, SMRT, and Sin3A (19). It has been shown recently that oligomerization and formation of HMW complexes by PML/RARα and AML-1/ETO play a critical role in the aberrant recruitment of HDAC activity (4, 20). The in vivo formation of HMW complexes is mediated by the PML and the ETO portion, leading to the oncogenic conversion of RARα and AML-1 in the respective leukemic fusion proteins PML/RARα and AML-1/ETO (4, 20). Comparably, PLZF/RARα also forms HMW complexes in vivo (21).

To answer the question of whether aberrant recruitment of HDAC by the AML-associated fusion proteins represents the only mechanism responsible for the leukemic differentiation block, we investigated the mechanism by which these translocation products block VitD₃-induced differentiation.

Here we report that the AML-associated translocation products block VitD₃-induced differentiation by a novel mechanism, sequestering the VitD₃ receptor, which is considered to be the key player in VitD₃ signaling.

MATERIALS AND METHODS

Cloning of the VDR ORF, PLZF/RARα Mutants, and Expression Vectors. VDR encoding cDNA was cloned from U937 cells. Three fragments were created by reverse transcription-PCR containing appropriate restriction...
DIFFERENTIATION BLOCK BY AML-ASSOCIATED FUSION PROTEINS

**A**

Fig. 1. A. modular organization of the APL-associated translocation products PLZF/RARα and PML/RARα, and their physiological counterparts PLZF, PML, and RARα. The major PLZF, PML, and RARα domains are shown with the respective known functions. NCR, nuclear corepressor complex (SMRT, Sin3, and N-CoR); SMRT, histone deacetylase 1; NLS, nuclear localization signal; TAD, transcriptional activation domain; DBD, DNA binding domain; LBD, ligand binding domain; AF-1 and AF-2, transactivation domains. Arrow, APL-breakpoints. B. modular organization of the AML2-associated translocation product AML2/ETO and its physiological counterparts, AML-1 and ETO. RHD, runt homology domain; TAF, TAF110 homology domain; HHR, hydrophobic heptad repeat; nery, nery homology domain; Zf, zinc finger domain.

**B**

**In Vitro Binding Assays, “Pull-Down” Assays, and Coimmunoprecipitation.**

GST-VDR, GST-N-CoR (aa 1792–2453), GST-SMRT (aa 982–1495), GST-Sin3A (aa 57–724), and GST-VDRE fusion proteins were expressed in *Escherichia coli* BL21 cells by induction with isopropyl-1-thio-β-d-galacto-pyranoside for 3 h at 37°C. Extracts were prepared after cell lysis by sonication/detergent treatment. Cell lysates were cleared by centrifugation (10 min at 10,000 × g) and incubated for 2 h at 4°C with glutathione–Sepharose beads (Amersham-Pharmacia Biotech). The beads were washed twice with E1A buffer [HEPES 50 mM (pH 7.8), NaCl 150 mM, EDTA 5 mM, DTT 1 mM, and NP-40 0.1% (v/v)] and quantified on SDS-PAGE electrophoresis by comparison to a standard of coupled T7/S6 transcription and translation kit was used according to manufacturer’s instruction (Promega). Five μl of in vitro translated 35S-labeled proteins in E1A buffer were incubated with GST or GST fusion proteins (10–μg) bound to glutathione Sepharose as described above for 1 h at 4°C. Beads were washed six times in the mentioned buffer. The bound proteins were eluted by boiling in 30 μl of 2× SDS-PAGE loading buffer, resolved by SDS-PAGE electrophoresis, and visualized by autoradiography.

For the pull-down experiments cells were resuspended in E1A buffer (NaCl 150 mM). Cell extracts were prepared after cell lysis by sonication and clarification by centrifugation (10 min at 10,000 × g). One mg of total protein was then incubated with GST or GST fusion proteins (10–μg) bound to glutathione Sepharose as described above for 1 h at 4°C. The bound proteins were eluted by boiling in 30 μl of 2× SDS-PAGE loading buffer, resolved by SDS-PAGE electrophoresis, and visualized by Western blotting with appropriate antibodies such as monoclonal mouse antihemagglutinin antibody (12CA5; Roche Diagnostics, Mannheim, Germany), polyclonal rabbit anti-PLZF, as well as anti-RARα (23), monoclonal mouse anti-PML (25), or monoclonal rat anti-VDR antibody (9A7; Biomol, Hamburg, Germany). Quantification of the immunoblots was performed by using the Quantity one 4.2.1. Software (Bio-Rad, Munich, Germany) on TIFF images of low or medium-density exposure X-ray films. For coimmunoprecipitation 293 cells were transfected with 5 μg of pCDNA3-PLZF, -PLZF#, as well as -PLZF/RARα constructs together with pCDNA-HA-VDR by calcium phosphate coprecipitation according to widely established procedures. The transiently transfected 293 cells were washed twice with PBS and collected in E1A buffer. U937 cells expressing HA-AML1-ETO were collected in the same buffer. The cell suspension was briefly sonicated and the lysates clarified by centrifugation. Lysates were precleared by incubation for 1 h with protein A-Sepharose (Amersham Pharmacia Biotechnology). Immunoprecipitation was obtained by adding to the precleared lysates the protein A-Sepharose and the relevant antibody. For anti-HA as well as anti-C protein immunoprecipitation an anti-HA and anti-C protein affinity matrices constituted by the rat monoclonal anti-HA antibody (clone 3F10) and anti-C protein (clone HPC4), respectively, covalently coupled to agarose beads (Roche), were used. The beads were washed five times in E1A buffer (150 mM NaCl) and resuspended in SDS sample buffer. Immunoprecipitation were detected by Western blotting with the indicated antibodies by the enhanced chemiluminescence method.

**Immunofluorescence.** Cells were applied to slides by cytocentrifugation, fixed with methanol (5° at room temperature), and permeabilized with (a) inducibility of protein expression upon Zn²⁺ treatment; (b) expression levels of the mutant proteins comparable with those of PLZF/RARα in the B412 clone; and (c) capacity of each clone to undergo U937-specific, Vdtd induction during the absence of Zn²⁺ induction. Expression of the respective transgenes was induced by 8–12-h exposure to 100 μM ZnSO₄ (Zn²⁺), and then evaluated by immunoblotting using the anti-RARα, anti-PML, or the anti-PML antibodies as reported previously (23, 24).

**Cell Differentiation.** Differentiation of HL-60 and U937 cell line under the influence of dihydroxy-Vitamin D₃, kindly provided by Dr. Hunziker, Hoffmann La Roche (Basel, Switzerland), was performed as described previously (6). Tri-chostatin A (Sigma, Deisenhofen, Germany) was added to the cell culture in a concentration of 1 μM 1 h before addition of Vdtd, t-RA (Sigma) was added to a final concentration of 10⁻⁶ M alone or in combination with Vdtd. After 48 h of treatment the percentage of differentiation antigen-positive cells and fluorescence intensity were evaluated by FACSscan (Becton Dickinson, Mountain View, CA) using appropriate antibodies such as CD11a, CD11b, and CD18 (BD Bioscience, Heidelberg, Germany) as described previously (6). In each sample viable cells were gated, and expression of surface markers of 5 × 10⁶ cells were evaluated.
acetic acid (2′ at 20°C). The monoclonal rat anti-VDR antibody (clone 9A7; Biomol; Ref. 26) and the polyclonal rabbit anti-RARα (24) were diluted 1:100 and incubated for at least 1 h, and after washing with PBS, the secondary antibodies, goat antirabbit FITC-conjugated and antirabbit Cy3-conjugated (Zymed Laboratories, San Francisco, CA; diluted to 1:100), were then incubated for 45 min. Antibody incubations were performed in 1% BSA, and washing was carried out in PBS containing 0.1% Tween 20. A short wash in H2O was carried out before the coverslips were mounted with Moviol (Sigma). Samples were examined in dual channels using a Leica TCS-NT confocal microscope, and images were processed using Leica TCS-NT V1.6.551 (Leica, Wetzlar, Germany) and Imaris 3 (Bitplane, Zürich, Switzerland) software.

RESULTS

Inhibition of HDAC Activity Increases VitD3-induced Differentiation in a Cell Type-specific Manner But Is Unable to Revert the PLZF/RARα-related Block of Differentiation. The mechanism by which unliganded VDR suppresses transcription is not completely clarified. It has been reported recently that VDR interacts with N-CoR (27) but not with SMRT (28). To investigate the potential of VDR to interact with members of HD-NCR, we incubated in vitro translated 35S-labeled VDR with GST fusion proteins of the domains of N-CoR (aa 1425–2543), SMRT (aa 982–1495), and Sin3A (aa 57–724) in the absence and in the presence of VitD3. Fig. 2A demonstrates that unliganded VDR bound to SMRT and N-CoR but not to Sin3A. The addition of VitD3 strongly decreased the affinity of this binding, suggesting that VDR interacts with members of HD-NCR in a ligand-dependent manner, comparable with RARs and TR.

HL-60 and U937 are hematopoietic precursor cell lines, which undergo monocytic differentiation on exposure to VitD3 (29, 30). We used these cells as a widely established model for the study of the VitD3-dependent pathway in hematopoiesis (31). To investigate the biological significance of the interactions between VDR and members of HD-NCR we tested whether inhibition of HDAC-activity: (a) increases sensitivity of cells to VitD3-induced differentiation; and (b) is able to overcome the PLZF/RARα-induced block of VitD3-differentiation. Therefore, we exposed HL-60 and U937 cells expressing PLZF/RARα fusion protein (under the control of the Zn2+-inducible MT-1 promoter) to VitD3 in the absence and presence of the HDAC inhibitor TSA. TSA was added at a concentration of 1 μM 1 h before the exposure to VitD3, and differentiation was assessed after 48 h. As control we used MT cells. In HL-60 MT cells TSA potentiated the ability of VitD3 to up-regulate expression of the CD11b and CD14 differentiation markers independently of the Zn2+ treatment (Fig. 2B). In HL-60 cells transfected with PLZF/RARα TSA increased VitD3-induced differentiation only in the absence of Zn2+. When expression of PLZF/RARα in these cells is induced by Zn2+ treatment, TSA only slightly increased VitD3-induced expression of CD11b and CD14 as compared with untreated and control cells (Fig. 2B). Surprisingly, in U937 cells VitD3-induced differentiation was not affected by the addition of TSA (data not shown).

Taken together these data indicate that the effect of HDAC inhibitor on the VitD3-induced differentiation pathway is cell-type specific and that TSA is not able to overcome the PLZF/RARα-related block of VitD3-induced differentiation in HL-60 cells, suggesting that the PLZF-RARα-induced block of VitD3 signaling is not because of its effect on histone-acetylation.

PLZF and PML, as well as the PLZF/RARα and PML/RARα Fusion Proteins, Bind VDR. To test the hypothesis of a direct influence of PLZF/RARα and PML/RARα on the activity of VDR we investigated the interaction between these fusion proteins and VDR. Physical interaction between wt RARα and VDR is known (32), but overexpression of wt RARα only induces a slight differentiation block in U937 cells as compared with that induced by PML/RARα or PLZF/RARα (6, 33). Thus, we studied the interaction between VDR and the partners of RARα involved in the t(11;17) and t(15;17) translocations.

To investigate a possible interaction between VDR and PLZF, as well as the PLZF-portion of PLZF/RARα (PLZF#), we cotransfected expression vectors for PLZF and PLZF#, respectively, together with HA-tagged VDR into 293 cells. The anti-HA antibody was able to precipitate a consistent fraction of PLZF as well as of PLZF# from the PLZF and PLZF# lysates, respectively (Fig. 3A). As control is reported the amount of HA-VDR precipitated by the anti-HA antibody (Fig. 3A).

To study the binding between VDR and PLZF as well as PLZF/RARα in U937 cells we performed pull-down experiments by incubating cell lysates of U937 cells expressing PLZF/RARα and PLZF, respectively, with the GST-VDR fusion protein coupled to Sepharose. After intense washing the complexes were dissolved in a SDS-PAGE and analyzed by Western blotting with the anti-PLZF antibody. GST-VDR was able to precipitate PLZF as well as PLZF/RARα from the cell lysates (Fig. 3B).

Fig. 2. A, interactions of the VDR with members of HD-NCR and with HD-1. In vitro translated VDR was precipitated with GST, GST-HD-1 (HD-1), GST-N-CoR (aa 1792–2453; N-CoR), GST-SMRT (aa 982–1495; SMRT), and GST-Sin3a (aa 57–724; Sin3a) proteins in the absence (−) and presence (+) of VitD3 (D3). The in vitro translated peptides were loaded as positive controls (1/10 of the amount that was used in the assay; 10% input). B, effect of TSA on VitD3-induced differentiation of HL-60 cells expressing PLZF/RARα. Differentiation levels induced by VitD3, in HL-60 cells transfected with the empty MT vector carrying the MT-1 promoter (MT) or expressing PLZF/RARα under the control of a Zn2+-inducible MT-1 promoter. The cells were analyzed in the presence (+) or absence (−) of Zn2+ induction of the transgene (Zn). VitD3-induced differentiation in the presence and absence of TSA is given as percentage of CD11b- and CD14-positive cells (results are given as the average of three independent experiments); bars, ±SD.

In summary, these data show that both APL-associated fusion proteins bind to VDR and that this binding is not mediated exclusively by their RARα portion. Moreover, the RARα translocation partners PML and PLZF bind to VDR.
Deletion of the RARα-DNA Binding Domain Abolishes the PLZF/RARα-related Block of VitD₃-induced Differentiation and Reduces Binding of PLZF/RARα to VDR. After establishing that PLZF, a potent transcriptional repressor, interacts with VDR, we investigated the role of PLZF# in the PLZF/RARα-related block of VitD₃-induced differentiation.

Thus we studied the biological significance of the binding between PLZF# and VDR for the induction of the differentiation block. Furthermore, we extended the analysis to the role of the RARα portion of the fusion protein for the blockage of VitD₃-induced differentiation. Therefore, we used clonal cell lines, which express the following constructs under the control of the Zn²⁺-inducible MT-1 promoter: (a) PLZF#, representing the PLZF portion of the fusion protein (clones B410 and B412); (b) PLZF/ΔCRARα, a PLZF/RARα deletion mutant lacking its RARα C-domain representing the DNA-binding domain (clones B44 and B412). These cell lines had a transgene expression level on Zn²⁺ exposure identical or higher than that of the recently described PLZF/RARα-expressing B412 clone used in this study (Ref. 6; data not shown). A MT U937 cell clone was used as a control. We exposed these cells to VitD₃ in the presence and absence of Zn²⁺-induced expression of the transgenes. The rate of VitD₃-induced differentiation was measured by quantitative FACS analysis of differentiation-specific surface markers (CD11a, CD11b, CD14, and CD18).

As shown in Fig. 4A the expression of PLZF# (clones B410 and B412) did not interfere with the differentiation potential of U937 cells. Moreover, the PLZF/ΔCRARα-expressing cells differentiated on VitD₃ exposure to the same extent as the control cells (MT). To investigate whether the inability of PLZF/ΔCRARα to block differentiation is because of differences in binding to VDR we performed pull-down experiments by incubating cell lysates of U937 cells expressing PLZF/RARα and PLZF/ΔCRARα, respectively, as described above. GST-VDR precipitated PLZF/RARα and less efficiently PLZF/ΔCRARα from the cell lysates, as determined by quantification of the bands with respect to the input (Fig. 4B).

In summary, these data provide evidence that binding of VDR to the PLZF portion of the leukemic fusion protein is not crucial for the block of VitD₃-induced differentiation in U937 cells. In contrast, the presence of the RARα-C domain retained in the fusion protein is indispensable for the PLZF/RARα-related block of VitD₃-induced differentiation in U937 cells most likely by interfering with the binding to VDR.

t-RA Restores the Differentiation Response to VitD₃ in PLZF/ RARα as well as in PML/RARα-expressing U937 Cells. Exposure of cells expressing the APL-associated fusion proteins to t-RA leads to degradation of both PLZF/RARα and PML/RARα (24, 34, 35). This degradation is because of caspase 3-like activity (34). Thus, we reasoned that treatment with t-RA might dissolve the binding between VDR and the APL-associated fusion proteins, thereby reestablishing sensitivity of PML/RARα, as well as of PLZF/RARα-expressing U937 cells to VitD₃-induced differentiation. To confirm this hypothesis we tested the influence of t-RA on the VitD₃-differentiation of U937 cells expressing PML/RARα or PLZF/RARα. These cells induced or not to express the transgene by Zn²⁺ were exposed to t-RA alone or in combination with VitD₃. Differentiation was measured after 48 h of treatment. As control we used the MT cells. Differentiation was assumed on up-regulation of CD14, a marker specific for monocytic differentiation induced by VitD₃ but not by t-RA (7, 33). As depicted in Fig. 3B, t-RA alone was able to induce a slight increase in the percentage of CD14-positive cells only in the PML/RARα-expressing cells but not in the MT control cells or in the PLZF/RARα-expressing cells. In contrast, exposure to t-RA decreased the extension of the PML/RARα and PLZF/RARα-related block of VitD₃-induced differentiation as revealed by the increase of CD14-positive cells as compared with cells treated with VitD₃ only (Fig. 4B).

Taken together these data show that most likely the presence of the translocation products is indispensable for the differentiation block in U937 cells.

The Expression of APL-associated Translocation Products Leads to an Impaired Localization of VDR. To confirm the biological relevance of the interaction between the APL-associated translocation products and VDR in vitro, we sought to disclose the mechanism by which this interaction interferes with the VitD₃-signaling pathway. Therefore, we studied the influence of the expression of PLZF/RARα as well as of PML/RARα on the localization of VDR in U937 cells by indirect immunofluorescence experiments.

Thus, we stained U937 cells induced to express PML/RARα or PLZF/RARα by Zn²⁺ treatment with an anti-VDR antibody. As control we used Zn (2)-treated MT U937 cells.

The anti-VDR-staining of the U937 MT cells as well as of wt U937
cells (data not shown) evidenced two to four nuclear foci for each cell (green fluorochrome in Fig. 5A). In contrast to the anti-VDR-staining of the PLZF/RARα and the PML/RARα-expressing U937 cells revealed only one to two foci and an additional micropunctated pattern in the nucleus (Fig. 5A).

Both PML/RARα and PLZF/RARα localize to so-called “microspeckles” in the nucleus (36–38). PML/RARα and PLZF/RARα colocalize to the same microspeckles when coexpressed in U937 cells (23). As the VDR localization pattern in the U937 cells expressing the APL-associated translocation products was very similar to the microspeckles of PML/RARα and PLZF/RARα, double-staining experiments were performed on the PML/RARα as well as on PLZF/RARα-expressing U937 cells using an anti-VDR monoclonal antibody and an anti-RARα polyclonal serum directed against the COOH-terminal RARα-F domain (24). MT cells were used as a control. The antibodies used did not cross-react in Western blotting on cell lysates from cells overexpressing VDR or PLZF/RARα (data not shown). As shown in Fig. 5B in MT control cells the superimposition of anti-VDR (Fig. 5B, green fluorochrome) and anti-RARα staining (Fig. 5B, red fluorochrome) revealed no colocalization between VDR and RARα. In the U937 cells expressing PML/RARα or PLZF/RARα, superimposition of anti-VDR and anti-RARα stainings disclosed that a fraction of VDR colocalized with both PML/RARα and PML/RARα microspeckles (Fig. 5B). In contrast only in a few PLZF/ΔCRARα-microspeckles was colocalization with VDR seen (Fig. 5B).

To investigate the influence of VitD3 on the colocalization between VDR and the APL-associated translocation products, we performed a double anti-RARα/anti-VDR staining of PML/RARα-expressing U937 cells in the presence and absence of VitD3-treatment. In MT control cells the superimposition of the anti-VDR (green fluorochrome in Fig. 5C) and the anti-RARα-staining (red fluorochrome in Fig. 5C) revealed no colocalization between VDR and RARα in the absence of VitD3. In the presence of VitD3, a partial colocalization between VDR and structures stained by the anti-RARα antibody was seen (Fig. 5C). In contrast, the colocalization between VDR and PML/RARα was not influenced by the presence of VitD3 (Fig. 5C). Images of PLZF/RARα-expressing cells gave identical results (data not shown).

Taken together these data show an in vivo interaction between VDR and APL-associated fusion proteins confirming our results obtained in the in vitro assays. Furthermore, these results strongly suggest that PLZF/RARα and PML/RARα delocalize a consistent portion of the VDR protein, probably resulting in a sequester of VDR.

Overexpression of VDR Overcomes the PLZF/RARα and PML/RARα-related Block of VitD3-induced Differentiation in U937 Cells. To confirm the hypothesis that VDR is sequestered by the APL-specific fusion proteins we studied the effect of the overexpression of VDR on the block of VitD3-induced differentiation by PML/RARα as well as PML/RARα. Thus, we overexpressed VDR in PML/RARα- and PML/RARα-positive U937 cells by retroviral infection. We used the bicistronic retroviral PINCO-vector (22) with VDR under the control of the long terminal repeat and the enhanced GFP driven by a cytomegalovirus promoter. For controls the cells were also infected with the empty PINCO, able to express only GFP. The infection rate ranged between 75% and 95% in three independent experiments (data not shown). Differentiation was measured by quantitative FACS analysis of differentiation-specific surface markers (CD11b and CD14) only on GFP-positive cells. As shown in Fig. 6, infection with the empty PINCO vector or PINCO-VDR did not interfere with the normal VitD3-induced differentiation of MT, PLZF/ RARα, or PML/RARα cells in the absence of Zn2+-induced expression of the transgenes. The empty PINCO did not influence the block of VitD3-induced differentiation in PLZF/RARα and PML/RARα-positive cells in the presence of Zn2+-induced expression of the transgenes. In the presence of Zn2+ the overexpression of VDR (PINCO-VDR) did not interfere with VitD3-induced differentiation in control cells, but in PLZF/RARα as well as in PML/RARα-expressing cells it led to a nearly complete rescue of differentiation with respect to cells infected with empty PINCO (Fig. 6).

Taken together these data indicate that the overexpression of VDR overcomes the block related to the expression of the APL-specific translocation products in U937 cells.

Overexpression of VDR Overcomes the Block of VitD3-induced Differentiation in U937 Cells Expressing the (8:21)-associated AML/ETO Fusion Protein. Another leukemia-related translocation product able to cause blockage of VitD3-induced differentiation in U937 cells is AML-1/ETO (9). Therefore, we addressed the question of whether overexpression of VDR also overcomes the differentiation block present in AML-1/ETO-expressing U937 cells. To study the effect of overexpression of VDR on VitD3-induced differentiation of AML-1/ETO-expressing U937 cells we infected U937 cells expressing HA-tagged AML-1/ETO under the control of the Zir2+-
inducible MT-1 promoter with the retroviral PINCO and PINCO-VDR vectors as described above. As controls we used the U937 MT cells and the PML/RARα/H9251-positive U937 cells.

As shown in Fig. 7, infection with the empty PINCO vector or PINCO-VDR did not interfere with the normal VD₃-induced differentiation of MT, PML/RARα, and AML-1/ETO cells in absence of Zn²⁺-induced expression of the transgenes. The empty PINCO did not influence the block of VD₃-induced differentiation in AML-1/ETO-positive cells in the presence of Zn²⁺-induced expression of the transgene. In the presence of Zn²⁺ the overexpression of VDR (PINCO-VDR) in AML-1/ETO-expressing cells led to a nearly complete rescue of differentiation in contrast to cells infected with empty PINCO (Fig. 7A).

To investigate whether AML-1/ETO binds to VDR, we first incubated in vitro-translated, ³⁵S-labeled AML-1 and ETO with a GST-VDR coupled to Sepharose beads. As shown in Fig. 7B, GST-VDR

Fig. 5. Influence of the expression of PLZF/RARα and PML/RARα on the localization of endogenous VDR. For clarity, results are reported only in presence of the Zn²⁺ induction of the transgene. MT cells with the empty MT vector. Cells were stained with the indicated antibodies: anti-VDR rat monoclonal antibody, green fluorochrome (α-VDR), anti-RARα polyclonal rabbit antibody, red fluorochrome (α-RARα). Colocalization images were obtained by electronic overlapping of the images (merge). Colocalization of fluorochromes yields a yellow color. A, localization of endogenous VDR in MT, PLZF/RARα, and PML/RARα-expressing U937 cells. Phase contrast images were underlayed. B, colocalization between the VDR and PLZF/RARα, PML/RARα, and PLZF/ΔCRARα. C, immunofluorescence analysis of the influence of exposure to VD₃ on the colocalization between PML/RARα and endogenous VDR.
precipitated in vitro translated AML-1 and much less efficiently ETO. To confirm the binding between VDR and AML-1/ETO, pull-downs were performed by incubating cell lysates of U937 cells expressing HA-tagged AML-1/ETO with the GST-VDR coupled on Sepharose. The complexes were dissolved in a SDS-PAGE and analyzed by Western blotting with an anti-HA-antibody. GST-VDR was able to precipitate a high amount of HA-tagged AML-1/ETO from the cell lysates (Fig. 7C).

To confirm the interaction between VDR and AML-1/ETO, we performed a coimmunoprecipitation experiment in U937 cells expressing the HA-tagged AML-1/ETO. As a negative control we used the anti-C protein antibody. As depicted in Fig. 7D, the anti-HA antibody was able to precipitate a high amount of HA-tagged AML-1/ETO from the cell lysates (Fig. 7C).

Taken together these results indicate that, comparable with PLZF or PLZF/RARα, the AML-1/ETO-related block of VitD3-induced differentiation is because of a sequester of VDR by the fusion protein most likely through the binding mainly to the AML-1 portion of the fusion protein.

DISCUSSION

One of the differentiation pathways blocked by AML-associated translocation products is that of VitD3-induced differentiation (6, 7, 9). Here we investigated the mechanisms by which the AML-associated translocation products, PML/RARα, PLZF/RARα, and AML-1/ETO, interfere with VitD3-induced differentiation.

On the basis of previously published data we started by investigating the role of aberrant recruitment of HDAC by the translocation products in the blockage of the VitD3-induced differentiation block in U937 cells. To clarify whether chromatin modeling plays the same role for VitD3 signaling as for T3- and t-RA-signaling, we studied the interaction between VDR, the receptor mediating VitD3-induced transcriptional activation, and the members of HD-NCR. Thus, we demonstrated that VDR binds to both N-CoR and SMRT but not to Sin3A in a ligand-dependent manner. This interaction between VDR and N-CoR is consistent with recently published data showing that binding to N-CoR depends on the ligand-dependent conformation of the VDR helix 12 (27). N-CoR and SMRT contain two receptor interaction domains (RID-1: aa 1055–1291 and RID-2: aa 1291–1495). In the absence of retinoid X receptor, VDR does not bind to separated SMRT RID-1 and RID-2 (28). The SMRT construct used in our study spans from aa 982–1495, containing both RID-1 and RID-2. This indicates that binding to VDR, in the absence of retinoid X receptor, requests the combined presence of SMRT RID-1 and RID-2. The biological significance of the interaction between VDR and HD-NCR in terms of recruitment of HDAC is proven by the fact that the inhibition of HDAC by TSA increases the sensitivity of HL-60 for VitD3-induced differentiation. To explain why neither TSA nor butyrate are able to increase the VitD3 sensitivity of U937 cells (data not shown), one could hypothesize the involvement of different or additional corepressors interacting with VDR such as Alien. It has been shown that Alien but not N-CoR displays selectivity for different VDRE structures, and that super-repression by Alien is affected only in part by TSA (39).

The fact that TSA was not able to overcome the PLZF/RARα-related blockade of VitD3-induced differentiation in HL-60 cells...
strongly suggests that aberrant recruitment of HDAC does not play a decisive role in the blockage of this differentiation pathway by the AML-associated translocation products.

Here we provide evidence that the physical interaction between VDR and the AML-associated translocation products is the cause of the blockage of VitD₃-induced differentiation in U937 cells expressing these fusion proteins. It is known that RARα can interact with VDR (40). But neither the overexpression of wt RARα nor of the RARα-portion of the fusion proteins is able to block VitD₃-induced differentiation to the same extent as the expression of PML/RARα or PLZF/RARα in these cells (6, 33). The fact that PML as well as PLZF and PLZF# are able to bind VDR suggest that both portions of the fusion proteins contribute to the physical interaction with VDR. Also taking into account that neither PML alone nor PLZF# are able to block VitD₃-induced differentiation proves that interference with VitD₃-signaling critically depends on the fusion of the translocation partners. This conclusion is corroborated by the observation that the RARα C-domain is the crucial domain regarding the capacity of the PLZF/RARα fusion protein to block VitD₃-induced differentiation. Our results on the role of the RARα C-domain are in accordance with the described function of the RARα C-domain in the biology of PML/RARα (33). The fact that the deletion of the RARα C-domain reduces strongly the interaction with VDR, reverts the de-localization of VDR, and abolishes the differentiation block by both APL-associated fusion proteins indicate that the C-domain mediates the interaction of the #RARα. The RARα C-domain represents the DNA-binding domain of RARα as well as of the APL-fusion proteins, and it remains to be clarified which role plays the DNA binding in the PML/RARα- and PLZF/RARα-related block of VitD₃-induced differentiation.

Our data presented here strongly suggest that the interaction between VDR and PLZF/RARα, as well PML/RARα, leads to sequestration of VDR. This sequestering of VDR is most likely responsible for the interference with VitD₃ signaling. This is proven by the findings that: (a) treatment of U937 cells expressing the APL-translocation products with t-RA nearly completely overcome the differentiation block without evidence of specific t-RA-induced differentiation; (b) the expression of PML/RARα and PLZF/RARα in U937 cells leads to delocalization of endogenous VDR, which is independent of the presence of VitD₃; and (c) an increase in the amount of VDR in the cells is sufficient to overcome the differentiation block. We excluded previously that VDR is down-regulated by the expression of PML/RARα, PLZF/RARα, or AML-1/ETO in U937 cells (Fig. 5; data not shown).

Regarding the interaction between VDR and PLZF/RARα, as well as PML/RARα, one could speculate that the effect of t-RA on VitD₃-induced differentiation is related to conformational changes in RARα, as shown for RARγ with a probable influence on the binding to VDR (41), or to the reported caspase-3-mediated degradation of the fusion proteins in the presence of t-RA. Here we demonstrate that the differentiation block related to the expression of the t(8;21)-derived AML-1/ETO fusion protein in U937 cells is abolished by overexpression of VDR. This suggests that the differentiation block caused by the sequestering of VDR is not limited to the APL-specific and RARα-derived translocation products but might represents a more general mechanism of blockage of differentiation. This is corroborated by the observation that the AML-1/ETO fusion protein strongly interacts with VDR.

In summary, the sequestration of VDR, the transcription factor that mediates VitD₃ signaling, represents a novel mechanism by which leukemia-associated translocation products block differentiation. It remains to be clarified whether in cells expressing the AML-associated translocation products the sequestration of transcription factors is a general mechanism inducing the blockage of differentiation.

REFERENCES


AML-associated Translocation Products Block Vitamin D₃-induced Differentiation by Sequestering the Vitamin D₃ Receptor

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