PML-RARα Alleviates the Transcriptional Repression Mediated by Tumor Suppressor Rb*

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A fusion between the promyelocytic leukemia (PML) protein and the retinoic acid receptor-α (RARα) results in the transforming protein of acute promyelocytic leukemia, PML-RARα. PML has growth-suppressive properties and is localized within distinct nuclear structures referred to as nuclear bodies. PML participates in numerous cellular functions, including transcriptional activation, apoptosis, and transcriptional repression, whereas PML-RARα blocks these functions. However, the role played by PML-RARα in leukemogenesis remains unclear. Here we report that PML is required for transcriptional repression mediated by the tumor suppressor Rb. Rb interacts with the histone deacetylase (HDAC) complex containing co-repressors and represses the transcription of the E2F target genes. Overexpression of PML enhanced Rb-mediated repression. The degree of Rb-mediated repression was weakened by injecting anti-PML antibodies and was lower in Pml−/− deficient mouse embryonic fibroblasts. PML-RARα inhibited Rb-mediated repression, and two co-repressor-interacting sites on the PML-RARα molecule were required for this activity. Furthermore, PML-RARα blocked the interaction between Rb and HDAC. Thus, aberrant binding of PML-RARα to co-repressor-HDAC complexes may inhibit their association with Rb, resulting in the abrogation of Rb activity. Thus, the disruption of Rb-mediated repression may be a contributory factor in leukemogenesis.

The retinoblastoma tumor suppressor protein (Rb)1 inhibits the G1/S transition in the cell cycle by repressing a subset of genes that are controlled by the E2F family (1, 2). Rb binds to the activation domain of E2F and actively represses the promoter by recruiting the histone deacetylase (HDAC) complex (3–5). Rb associates not only with HDAC1 (3–5) but also with the co-repressors Ski and mSin3A (6). Co-repressors N-CoR/SMRT, mSin3A, and Ski/Sno interact with each other, and all of these co-repressors are required for the transcriptional repression mediated by Mad and thyroid hormone receptor β (TRβ) (7–12). The N-CoR or SMRT complex contains the class II HDAC (13–15), while the mSin3 complex contains the class I HDAC (16). The mSin3 complex also contains two Rb-associated proteins, RbAP46 and RbAP48 (16), which directly interact with histone H4 (17). These results suggest that Rb represses the E2F target gene by recruiting multiple complexes containing both class I and II HDACs and co-repressors N-CoR/SMRT, mSin3, and Ski/Sno. In fact, we recently demonstrated that loss of one copy of sno leads to up-regulation of a target gene of Rb, cdc25A, and increases susceptibility to tumorigenesis in mice (18).

The promyelocytic leukemia (PML) protein was originally identified as a fusion partner of the retinoic acid receptor-α (RARα) in the transforming protein (PML-RARα) found in acute promyelocytic leukemias (19–21). However, further analysis of PML indicated that PML has a capacity to suppress cellular proliferation (22, 23). PML has the RBCC motif consisting of a Cys-rich zinc finger (RING finger) motif and two Cys-rich regions (B-boxes), followed by a coiled-coil region. Wild-type PML is localized to nuclear dot-like structures known as PML nuclear bodies (PML NBs), which are normally comprised of 5–30 discrete domains (24–26). PML-RARα alters the dot-like NB structure into numerous small speckles, and normal NB structure can be regained by treating the cells with all-trans-retinoic acid (RA). Although PML appears to be involved in multiple functions, including apoptosis and transcriptional activity (reviewed in Ref. 27), we recently demonstrated that PML directly associates with multiple co-repressors (N-CoR/SMRT, mSin3A, and Ski) and that it is required for Mad-mediated transcriptional repression (28). Unlike PML, which directly interacts with co-repressors through its coiled-coil domain, PML-RARα bears two sites that interact with the co-repressors: one is the coiled-coil region on PML and the other is the CoR box on RARα. Via these two sites, PML-RARα may aberrantly bind to the co-repressor-HDAC complex, leading to the Mad-mediated repression. We were interested in determining whether PML/PML-RARα is specific for Mad-mediated silencing or whether this is a common feature of other repressors that utilize co-repressors such as Ski.

Here, we report that PML is required for Rb- and TRβ-mediated silencing and that PML-RARα inhibits this silencing. Inhibition of the activity of the tumor suppressor Rb may be an important mechanism for PML-RARα-induced leukemogenesis.

1 The abbreviations used are: Rb, retinoblastoma gene product; HDAC, histone deacetylase; TRβ, thyroid hormone receptor β; PML, promyelocytic leukemia; RAR, retinoic acid receptor-α; NB(α), nuclear body(ies); RA, retinoic acid; MEF, mouse embryonic fibroblast.

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MATERIALS AND METHODS

Subcellular Localization of Rb and PML—Immunostaining of endogenous Rb and endogenous PML in NB4 cells were performed with anti-Rb mouse monoclonal antibody (G3–245, PharMingen) and anti-PML rabbit polyclonal antibodies (28) after treatment of the cells with RA (1 μM) for 3 days. These procedures and confocal microscopy were performed as described previously (12).

Effect of PML on Rb- and TRβ-mediated Repression—CV-1 cells (1.5 × 10^5 cells per six-well plate) were transfected using LipofectAMINE with a mixture containing the Gal4 site-containing luciferase reporter (1 μg) and either Gal4-Rb, Gal4-TRβ (0.01 μg), Gal4TRβ (0.05 μg), or the Gal4 expression plasmid (0.01 or 0.05 μg) together with the PML expression plasmid pM-L (0.25 or 0.5 μg) and the internal control plasmid pRL-TK (Promega) (0.25 μg). The total amount of plasmid DNA was adjusted to 3 μg by addition of the control plasmid DNA lacking the cDNA. After transfection, cells were cultivated using charcoal-treated serum, and dual luciferase assays were then performed. Similar experiments were performed using the Gal4-3EF1 (0.03 μg) expression plasmid.

Single-cell Microinjection Assay—Rabbit polyclonal antibodies raised against GST-PML were purified using antigen columns. Microinjection assays were performed using Rat-1 cells as described previously (12).

Repressor Activity of Rb and TRβ in Mouse Embryonic Fibroblasts (MEFs)—Wild-type and Pml–/– MEFs (1.5 × 10^5 cells per six-well plate) were transfected using LipofectAMINE with a mixture containing the Gal4 site-containing luciferase reporter (1.5 μg) and either Gal4-Rb, Gal4-TRβ, the control Gal4-expression plasmid or Gal4-trf1, and the internal control plasmid pRL-TK (0.5 μg). Dual luciferase assays were performed as described above.

Effect of PML-RARα on the Rb- and TRβ-mediated Repression—CV-1 cells (3 × 10^5 cells per 60-mm dish) were transfected by the CaPO4 method with a mixture containing the Gal4 site-containing luciferase reporter (1.5 μg) and either 2.5 μg each of Gal4-Rb, Gal4-TRβ, or the Gal4 expression plasmid (0.25 or 0.5 μg) together with the plasmid pM-L-RARα (0.5 or 1.0 μg), which expresses various forms of PML-RARα, and the internal control plasmid pRL-TK (0.5 μg). Dual luciferase assays were then performed. The total amount of plasmid DNA was adjusted to 6 μg by addition of the control plasmid DNA lacking the cDNA. Similar experiments were performed using Gal4-3EF1 (0.03 μg) expression plasmid. RA (0.8 nM) treatment was performed for 30 h before lysis preparation.

Effect of PML-RARα on E2F-dependent Transcription—A mixture containing 0.1 μg of the E2F sites containing luciferase reporter, 0.1 μg of the E2F1 expression plasmid or the control plasmid, and 1.5, 2.0, or 2.5 μg of the PML-RARα expression plasmid together with 0.1 μg of the internal control plasmid pRL-TK was transfected into CV-1 cells (3 × 10^5 cells per 60-mm dish) using LipofectAMINE, and luciferase assays were performed. The total amount of plasmid DNA was adjusted to 5 μg by addition of the control plasmid DNA lacking the cDNA.

Co-immunoprecipitation—To study the interaction between Gal4-Rb and HDAC1, CV-1 cells (6 × 10^5 cells per 100-mm dish) were co-transfected using LipofectAMINE with Gal4-Rb or the Gal4 expression plasmids pCMV-Gal4-Rb or pCMV-Gal4 (2 μg), the FLAG-HDAC1 expression plasmid pact-FLAG-HDAC1 (2 μg), the PML-RARα expression plasmid pact-PML-RARα (3 μg), and the internal control plasmid pRL-TK (0.1 μg). Forty hours after transfection, cells were lysed by sonication in LSLD buffer (50 mM HEPES (pH 7.4), 50 mM NaCl, 0.1% Tween 20, 20% glycerol), and immunoprecipitation was performed with anti-Gal4 monoclonal antibody (Upstate Biotechnology). Western blotting was performed using anti-FLAG antibody.

For co-immunoprecipitation of endogenous Rb and HDAC proteins of NB4 cells, cells were treated with RA (1 μM) or control solvent for 70 h. Lysates were prepared by mild sonication in LDDL buffer. Anti-Rb rabbit antibodies (C-15, Santa Cruz) were used for immunoprecipitation. The immunocomplexes were washed with TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) and used for Western blotting with anti-HDAC1 (C-19, Santa Cruz) and anti-HDAC2 (C-19, Santa Cruz) goat polyclonal antibodies.

RESULTS

As reported previously by Alcalay et al. (29), RB was found to co-localize with PML. For this purpose, we used APL-derived NB4 cells, which express PML-RARα and undergo differentiation after RA treatment (Fig. 1A). RA treatment of NB4 cells induces degradation of PML-RARα, resulting in the recovery of the normal dot-like structures of NBs containing PML. In RA-treated NB4 cells, endogenous Rb and PML co-localized to NBs.

![Fig. 1. PML co-localizes with Rb and enhances Rb-mediated transcriptional repression. A, co-localization of endogenous PML and endogenous Rb. NB4 cells were treated with RA for 3 days, immunostained with the antibodies against the protein shown above, and analyzed by confocal microscopy. In the left two panels, Rb and PML staining visualized by rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. In the panel indicated as Overlay, the signals for both proteins are superimposed. More than 95% of 300 cells scored exhibited the similar pattern. B, enhancement of Rb- and TRβ-mediated repression by PML. CV-1 cells were transfected with the Gal4 site-containing luciferase reporter together with either Gal4-Rb, Gal4-TRβ, Gal4-3EF1, or Gal4 expression plasmid and either 0.75 (+) or 1.5 μg (+ +) of the PML expression plasmid. Luciferase activity was then measured. The black bar indicates the data obtained when PML was co-expressed. The data are averages of the results of three experiments. S.D. values are shown.

We observed that some PML signals did not co-localize with Rb. This might be explained by the association of a proportion of PML molecules with co-activator such as CBP.

The physical association of PML with Rb raised the possibility that PML might be important for transcriptional repression mediated by Rb. To investigate this, we first examined the effect of overexpression of PML on Rb-mediated repression (Fig. 1B). The Gal4-Rb fusion, which consists of the Gal4 DNA-binding domain and the repressor domain of Rb, repressed transcription from a Gal4 site-containing reporter. This Gal4-Rb-induced repression was further enhanced by PML. Similar results were obtained with the Gal4-TRβ. This effect of PML was specific to Rb and TRβ, since PML did not enhance the repression by another repressor ΔEF1, whose repressor activity was previously shown not to be mediated by the co-repressor c-Ski (12).

Micro-injection experiments were then performed using a Gal4-lacZ reporter containing Gal4-binding sites (Fig. 2A). Injection of the reporter into Rat-1 cells gave rise to many lacZ-positive cells, whereas co-injection of the lacZ reporter with the plasmid encoding Gal4-Rb or Gal4-TRβ resulted in a decrease in the number of lacZ-positive cells. This decrease was partially relieved by co-injection with anti-PML antibody. The effect of the anti-PML antibody could be blocked by co-injection with the PML expression plasmid. In contrast, anti-PML antibody did not affect repression by the Gal4-3EF1 fusion, whose repressor activity does not require the c-Ski complex. The incomplete abrogation of the Gal-Rb and Gal4-TRβ function by anti-PML antibody may be due to the presence of other PML-related protein(s) containing the RBCC motif.

To further confirm that PML is involved in Rb- and TRβ-mediated repression, we used MEFs from a Pml-deficient mouse (Fig. 2B). The degree of repression by Gal4-Rb or Gal4-
TRβ in Pml−/− cells was lower than that in wild-type cells, whereas the repressor activity of Gal4-δEF1 was similar in both types of cells. The incomplete abrogation of repression mediated by Gal4-Rβ or Gal4-TRβ in Pml−/− cells may be due to the presence of PML-like proteins containing the RBCC motif.

As we recently reported (28), PML-RARα inhibits Mad-mediated repression. This activity of PML-RARα required the presence of two sites that interact with the co-repressors: one is the coiled-coil region on PML, and the other is the CoR box on RARβ. Since PML is also required for Rb-mediated repression, we examined this by co-immunoprecipitation (Fig. 4A). The results described above suggest that an interaction between Rb and two sites on PML-RARα blocks the interaction between Rb and the co-repressor-HDAC complex. We further confirmed the effect of PML-RARα on Rb activity (Fig. 3B). Co-transfection of this reporter into CV-1 cells together with small amount of the E2F1 expression plasmid slightly enhanced the luciferase expression. This suggested that E2F binds to specific sites on the promoter to enhance transcription from the reporter. Co-expression of PML-RARα with this repressor and the E2F1 expression plasmid enhanced the luciferase expression in a dose-dependent manner, indicating that PML-RARα inhibits the activity of endogenous Rb. These results indicate that PML-RARα inhibits Rb-dependent transcriptional repression.

The results described above suggest that an interaction between Rb and two sites on PML-RARα mediates inhibition of Rb activity. One possibility is that PML-RARα blocks the interaction between Rb and the co-repressor-HDAC complex. We examined this by co-immunoprecipitation (Fig. 4A). The plasmids expressing FLAG-HDAC1 and Gal4-Rβ were transfected into 293T cells together with the PML-RARα expression plasmid and increasing amounts of PML-RARα expression plasmid was transfected into CV-1 cells, and luciferase activity was measured. The amount of PML-RARα expression plasmid was 1.5 (++) or 2.0 (+++), or 2.5 µg (++++). An average of three experiments ± S.E. is shown.

To directly investigate the effect of PML-RARα on the interaction between endogenous Rb and HDAC, we performed co-immunoprecipitation experiments using RA-treated and untreated NB4 cells (Fig. 4B). NB4 cells express PML-RARα, whereas RA treatment of NB4 cells induces degradation of PML-RARα. Anti-Rb antibody co-precipitated the endogenous HDAC1 and HDAC2 proteins in the lysates from RA-treated NB4 cells.
but not from lysates of untreated cells. Similar amounts of endogenous Rb were precipitated by anti-Rb antibody from RA-treated and untreated NB4 cells. These results suggest that PML-RARα inhibits the association between Rb and HDAC.

**DISCUSSION**

Our results indicate that PML and PML-RARα have opposite effects on Rb-mediated transcriptional repression; PML stimulates Rb-mediated transcriptional repression, whereas PML-RARα inhibits Rb activity. The presence of two sites on the PML-RARα molecule, which interact with the co-repressors, is required for the inhibition of Rb activity. This suggests that the binding of PML-RARα to the co-repressors complexes induces a conformational change in the complexes, which blocks the interaction between Rb and HDAC. Since we recently demonstrated that PML-RARα also blocks Mad-mediated repression, the activity of two tumor suppressors, Mad and Rb, are probably inhibited through this mechanism. Thus, our results suggest an important role for Rb in acute promyelocytic leukemia. Consistent with this, it was reported that Rb protein is defective in binding to a viral oncprotein in the M3 promyelocytic subtype of acute myeloid leukemia patients (30).

Rb function was only partly abrogated by injection of anti-PML antibody and only partly diminished in PML-RARα mouse embryos (30). Since RFP was reported to associate with PML NBs (32), RFP-RET fusion oncprotein may also affect Mad and Rb activities like PML-RARα. At present, relatively little is known about how PML acts in transcriptional repression. However, one hypothesis is that PML and possibly other RBCC motif-containing proteins may act as key factors, such as scaffold proteins, to maintain the correct architecture or subcellular localization of co-repressors-HDAC complexes. Thus, PML and PML-RARα may have the capacity to affect the activity of many other repressors.

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