Aberrant Association of Promyelocytic Leukemia Protein–Retinoic Acid Receptor-α with Coactivators Contributes to Its Ability to Regulate Gene Expression

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The aberrant association of promyelocytic leukemia protein–retinoic acid receptor-α (PML–RARα) with coactivator complexes is generally thought to contribute to the ability of PML–RARα to regulate transcription. We report here that PML–RARα acquires aberrant association with coactivators. We show that endogenous PML–RARα interacts with the histone deacetyltransferases CBP, p300, and SRC-1 in a hormone-independent manner, an association not seen for RARα. This hormone-independent coactivator binding activity requires an intact ligand-binding domain and the NR box of the coactivators. Confocal microscopy studies demonstrate that exogenous PML–RARα sequesters and colocalizes with coactivators. These observations correlate with the ability of PML–RARα to attenuate the transcription activation of the Notch signaling downstream effector, CBFI1, and of the glucocorticoid receptor. This includes attenuation of the glucocorticoid-induced leucine zipper (GILZ) and FLJ25390 target genes of the endogenous glucocorticoid receptor. Furthermore, treatment of NB4 cells with all-trans-retinoic acid, which promotes PML–RARα degradation, resulted in increased activation of GILZ. On the basis of these findings, we propose a model in which the hormone-independent association between PML–RARα and coactivators contributes to its ability to regulate gene expression.

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Acute promyelocytic leukemia (APL) is a disease in which a terminal differentiation block of myeloid precursors occurs at the promyelocytic stage of development (1). APL pathogenesis has been attributed to aberrant signaling due to a common chromosomal translocation involving the retinoic acid receptor-α (RARα) gene on chromosome 17q21 (2). This chromosomal translocation results in two reciprocal fusion genes that are translated into reciprocal fusion proteins found to be oncogenic. There are five known proteins that create fusions with RARα: promyelocytic leukemia (PML), promyelocytic leukemia zinc finger (PLZF), nuclear matrix-associated (NuMA), nucleophosmin (NPM), and signal transducer and activator of transcription 5β (Stat5β) (2–7). Among them, >90% of APL patients express a fusion with PML to generate RARα-PML and PML–RARα.

Disruption of the retinoid signaling pathway is a key pathogenic feature of APL (8–10). RARs are members of the nuclear receptor family that controls processes such as development, differentiation, and homeostasis through regulation of complex gene networks. RARs form heterodimers with retinoid X receptors (RXRs) and bind to DNA sequences harboring direct repeats, (A/G)(G/T)TCA, separated by 5 bp. Transcription regulation by RXR/RAR heterodimers involves the exchange of corepressor and coactivator complexes, which are controlled by the hormone binding status of the receptor (11, 12). Unliganded RXR/RAR heterodimers bind corepressor complexes to inhibit transcription, whereas hormone-bound RXR/RAR heterodimers dissociate from the corepressors and concomitantly recruit the coactivator complexes, leading to transcription activation. The best known corepressors include SMRT and the nuclear receptor corepressor, which form complexes with mSin3A and histone deacetylases (13, 14), leading to deacetylation of histone tails to generate compact chromatin, thus inhibiting the general transcription machinery from reaching the promoter. The coactivators include members of the p160 family, SRC-1, ACTR/RACK1/pCIP/SRC-3, and TIF2/GRIPI, as well as the potent histone deacetyltransferases PCAF and p300/CBP (15–22). In contrast to the action of histone deacetylases, histone acetyltransferases acetylate histone tails, resulting in weaker associations of histones with chromatin and creating a local environment conducive for binding of the general transcription machinery to the target promoter. These notions are...
consistent with the observations that histone acetylation is linked to transcription activation and that deacetylation is associated with transcription repression.

Molecular and structural studies of the nuclear receptor ligand-binding domain (LBD) of RXRα indicate that, in the absence of hormone, helix 12 of the LBD is held in a position extended away from the rest of the LBD (23). In this conformation, corepressors bind receptors via a common motif, [(I/L)XX(I/V)I] (where X is any amino acid), which is known as the CoRNR box (24–26). Hormone binding induces a conformational change in which helix 12 folds back to contact helices 3–5 of the LBD (23, 27). Consequently, such conformational changes result in dissociation of the corepressors and recruitment of the coactivators via the LXXLL motif present in the coactivators (28, 29). It has been proposed that these consensus motifs signify a common mechanism of transcription regulation throughout the nuclear receptor family members.

The mechanisms underlying transcription regulation by PML-RARα appear to be more complex than those of RXRa/RARα heterodimers. In vitro studies have shown that, in addition to its ability to form heterodimers with RXRa, PML-RARα is capable of forming homodimers (30, 31) that bind corepressors more tightly than does RXRa/RARα and that require retinoic acid concentrations higher than physiological levels to dissociate from corepressors (≥1 μM) (2, 5, 31). Therefore, at physiological concentrations of retinoic acid (10–100 nM), PML-RARα acts as a dominant-negative inhibitor of RARs, leading to a constitutive repression of RAR target genes. This model is supported by transient transfection assays, in which PML-RARα can potently repress transcription when it is tethered to the yeast Gal4 DNA-binding domain (32). There is also evidence suggesting that PML-RARα can promote formation of a heterochromatin-like structure to keep target genes repressed through recruitment of histone methyltransferases such as SUV39H1 (33). However, there is no in vivo evidence to suggest that PML-RARα homodimers compete with RXRa/RARα for DNA binding. Furthermore, recent reports suggested that overexpression of PML-RARα leads to a reduction of nuclear receptor corepressor protein levels (34, 35). Likewise, other studies have indicated that, in the absence of hormone, PML-RARα activates retinoic acid response element-mediated reporter activity in the absence of retinoic acid (36, 37). This differential regulation of transcription is likely promoter- and cell type-specific; however, it highlights the fact that a simple model of constitutive repression of RARα target genes by PML-RARα is not sufficient to explain all of its possible roles in transcription regulation.

Although the aberrant association of PML-RARα with corepressors is well characterized, interactions with coactivators have not been well explored, although one study with NPM-RARα suggests that this fusion protein may interact with coactivators in an aberrant manner compared with wild-type RXR/RARα (38). In this study, we show that PML-RARα homodimers acquire the ability to interact with coactivators in a hormone-independent manner. Consistent with these data, we found that ectopic expression of PML-RARα in the absence of hormone is able to induce mislocalization of some coactivators to a non-nuclear compartment. We also found that overexpression of PML-RARα decreases transcription activation by the glucocorticoid receptor (GR) and CBF1. In light of these findings, we propose a distinct mechanism by which PML-RARα regulates transcription not only as a repressor of RARα target genes, but as a factor capable of repressing the activation of a subset of genes whose activation relies on coactivator recruitment.

**EXPERIMENTAL PROCEDURES**

Plasmid Construction—The plasmids pCMX, pCMX-PML(S)-RARα, pCMX-TAN-1, pCBF1-TK-Luc, pCMX-GRα, pGRE-TK-Luc, pCMX-ERα, pERE-TK-Luc, pCMX-MEF2C, and pMEF2-TK-Luc have been described previously (39–42). pCMX-FLAG-ACTR, pGEX4T-1-ACTR(RID), pBG79-CBP, pCMX-HA-PML, pCMX-HA-RARα, pCMX-HA-PML(S)-RARα, pCMX-HA-PLZF, pCMX-HA-PLZF-RARα, pCMX-FLAG-PCAF, pGAD-RARα(LBD), and pGAD-PML(S)-RARα were generated by PCR of the corresponding cDNA and subcloning into the indicated vector.

Yeast Methods—Yeast two-hybrid assays were carried out according to the protocol of Clontech. Where indicated, yeast cells were grown overnight (16–24 h) in medium containing all-trans-retinoic acid (ATRA). Liquid β-galactosidase activity was assayed, and the values were derived from duplicate experiments with two independent clones.

Luciferase Reporter Assays—Transient transfection assays were carried out according to our published protocol (43). CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate at 37 °C in 7% CO₂. For reporter assays, cells (85–95% confluence, 48-well plates) were cotransfected with equal amounts of the corresponding pCMX constructs, not exceeding 1 μg, as well as 100 ng of pCBF1-TK-Luc, pGRE-TK-Luc, pERE-TK-Luc, or pMEF2-TK-Luc and 100 ng of pCMX-LacZ in 200 μl of Optimem 1 using the transfection reagent Lipofectamine 2000 following a procedure adapted from Invitrogen. CV-1 cells were transfected, washed, and placed in Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. After 24 h, the medium was replaced or not with 1 μM dexamethasone for GR assays or with 1 μM estradiol for estrogen receptor-α assays or grown in regular Dulbecco’s modified Eagle’s medium and non-stripped fetal bovine serum for TAN-1 assays. Cells were harvested and assayed for luciferase and β-galactosidase activities 36–48 h after transfection. Luciferase activity was normalized to the level of β-galactosidase activity. Each transfection was performed in triplicate and repeated at least three times.

Electroporation of HL-60 cells—HL-60 cells were grown in RPMI 1640 medium supplemented with 10% charcoal-stripped bovine calf serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate at 37 °C in 7% CO₂. For transfection, 7–10 × 10⁶ cells were spun down and resuspended in 200 μl of OptiMEM I. 60 μg of hemagglutinin (HA)-PML-RARα expression plasmid was added, and the mixture was placed into a 0.2-mm gap electroporation cuvette. The cells were electroporated with a 140-V square-wave pulse for 0.25 ms using the Bio-Rad Gene
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Pulser system. Following this, the cells were grown 24 h in RPMI 1640 medium and then harvested for immunofluorescence microscopy.

Immunoprecipitation—To detect endogenous interactions, NB4 cells were grown in RPMI 1640 medium supplemented with 10% charcoal-stripped bovine calf serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin sulfate at 37 °C in 7% CO₂. Lysates were made using NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothreitol) with mixture of protease inhibitors (Sigma) and lysed by sonication. The resulting lysates were immunoprecipitated in NETN buffer for 4 h with 4 µg of antibody (anti-RARα (C-20), anti-SRC-1 (M-341), anti-p300 (N-15), or anti-CBP (A-22); Santa Cruz Biotechnology, Inc.). The immunoprecipitated complexes were resolved by SDS-PAGE and immunoblotted overnight with the antibodies indicated in Fig. 4 (anti-RARα, anti-SRC-1, anti-p300, anti-CBP, or anti-PML (H-238)) at a 1:500 dilution in 1× phosphate-buffered saline (PBS)/Tween 20 at 4 °C. Human embryonic kidney 293 cells were transfected with 10 µg of DNA composed of pCMX-FLAG-PCAF, pCMX-FLAG-ACTR, pCMX-HA-PML-RARα, pCMX-RARα, or pCMX alone using Lipofectamine 2000 following the published protocol from Invitrogen. 48 h after transfection, whole cell lysates were made using radioactive precipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) plus protease inhibitors and then immunoprecipitated in radioactive precipitation assay buffer using red anti-FLAG antibody M2 affinity gel (Sigma) for 4 h at 4 °C. The immunoprecipitates were analyzed by Western blotting using anti-FLAG (Sigma) and HA-conjugated anti-horseradish peroxidase (Roche Applied Science) antibodies (1:1000 dilution in 1× PBS/Tween 20 for 1 h at room temperature). The corresponding secondary antibodies were used, and visualization of the products was done using an ECL detection kit (Pierce). For immunoprecipitations done in the presence of hormone, cells were grown for 12 h in medium with charcoal-stripped serum supplemented or not with 1 µM ATRA. ATRA was also included in the immunoprecipitation reactions and wash buffer at the same concentration.

Immunofluorescence Microscopy—CV-1 and HL-60 cells were transfected with an expression plasmid of HA-PML-RARα. Transfected cells were grown in medium containing stripped serum for an additional 24–48 h. For immunofluorescence microscopy, transfected cells were fixed in 3.7% paraformaldehyde in 1× PBS for 30 min at room temperature and permeabilized in 1× PBS with the addition of 0.1% Triton X-100 and 10% goat serum for 10 min. The cells were washed three times with 1× PBS and incubated in a solution of PBS, 10% goat serum, and 0.1% Tween 20 (buffer A) for 60 min. Incubation with primary antibodies was carried out for 30 min in buffer A. The cells were washed three times with 1× PBS, and the secondary antibodies were added for 30–60 min in the dark at room temperature in buffer A. Coverslips were mounted on slides using VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (H-1200, Vector Laboratories). Imaging was performed on a Leica Model DMLB microscope, and pictures were taken with a SPOT camera using SPOT Advanced software (Diagnostic Instruments, Inc.). The primary antibodies used were as follows: mouse anti-HA monoclonal, rabbit anti-p300 polyclonal (N-15), rabbit anti-CBP polyclonal (A-22), and rabbit anti-SRC-1 polyclonal (M-341) (all from Santa Cruz Biotechnology, Inc.). The secondary antibodies used were Alexa Fluor 594-conjugated anti-mouse and Alexa Fluor 488-conjugated anti-rabbit antibodies (Molecular Probes).

Confocal Microscopy—All confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A ×63 numerical aperture of a 1.4 oil immersion Plan Apochromat objective was used for all experiments. To investigate the localization of transiently transfected HA-PML-RARα, images of Alexa Fluor 488 were collected using a 488-nm excitation light from an argon laser, a 488-nm dichroic mirror, and 500–550-nm band pass barrier filter. For endogenous coregulator anti-SRC-1, anti-CBP, and anti-p300 antibodies, images of Alexa Fluor 594 were collected using a 633-nm excitation light from a HeNe laser, a 633-nm dichroic mirror, and 650-nm long pass filter. All 4’,6-diamidino-2-phenylindole-stained nuclear images were collected using a Coherent Mira-F-V5-XW-220 (Verdi 5W) Ti:sapphire laser tuned at 750 nm, a 700-nm dichroic mirror, and a 390–465-nm band pass barrier filter.

Glutathione S-Transferase (GST) Pulldown Assays—For GST pulldown assays, in vitro translated HA-RARα, HA-PML, HA-PML-RARα, HA-PLZF-RARα, or HA-PLZF was incubated for 30 min on a nutator at room temperature with GST-tagged ACTR receptor interaction domain (RID)-conjugated glutathione-Sepharose beads in NETN buffer with a mixture of protease inhibitors (Sigma) in the presence or absence of ATRA (10–1000 nM). After incubation, the beads were washed three times with NETN buffer and collected by centrifugation. The proteins were eluted and denatured by placing the samples at 100 °C for 5 min and then run on 10% SDS-polyacrylamide gels. The products were visualized by Western blot analysis with HA-conjugated anti-horseradish peroxidase antibody.

Reverse Transcription (RT)-PCR—A549 cells were transfected with HA, HA-PML-RARα, or HA-PML-RARα(F584A) using Lipofectamine 2000. Where indicated, samples were treated for 16–20 h with 1 µM dexamethasone before harvesting. One-third of the cells were used to make whole cell lysates using radioimmuneprecipitation assay buffer plus protease inhibitors. The other two-thirds of the cells were used for RNA isolation. NB4 cells were grown in RPMI 1640 medium supplemented with 10% charcoal-stripped fetal bovine serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin sulfate at 37 °C in 7% CO₂. They were either left untreated or treated alone or in combination with 1 µM ATRA for 72 h and 1 µM dexamethasone for 16–18 h. RNA isolation was performed using an RNeasy mini RNA isolation kit (Qiagen Inc.). All procedures were performed according to the manufacturer’s protocol. DNase I digestion was performed on an RNeasy column following the manufacturer’s protocol using RNase- and DNase-free DNase I (Qiagen Inc.). The isolated RNA was used in a semiquantitative RT-PCR using a one-step RT-PCR kit (Invitrogen) according to the manufacturer’s protocol. 500 ng of RNA template was used in each reaction, and PCR amplification was repeated for 40 cycles. The final primer concentra-
of hormone. HA-RARα binding to GST-ACTR(RID) was measured by GST pulldown assays. Immobilized purified GST-ACTR(RID) was incubated with *in vitro* translated HA-PML-RARα in the absence or presence of 1 μM ATRA. Bound complexes were eluted and analyzed by Western blot analysis with anti-HA antibody. B, RARα only associates with ACTR(RID) in the presence of hormone. HA-RARα binding to GST-ACTR(RID) was monitored by GST pulldown assays as described for A using *in vitro* translated HA-RARα. C, PML does not bind ACTR(RID). HA-PML binding to GST-ACTR(RID) was monitored by GST pulldown assays as described for A using *in vitro* translated HA-PML. D, PML-RARα binding to SMRT requires 1 μM ATRA to induce dissociation. HA-PML-RARα binding to GST-SMRT(RID) was monitored by GST pulldown assays as described for A using increasing amounts of ATRA (10 nM to 1 μM).

### Results

The observations that PML-RARα requires higher concentrations of hormone to dissociate from corepressors and that both corepressor- and coactivator-binding domains overlap in the modeled nuclear receptor LBD suggested that PML-RARα may interact with both sets of coregulators differently from wild-type RARα (23). To investigate this possibility, we characterized the association of PML-RARα with coactivators in solution by GST pulldown experiments. A fusion protein composed of GST attached to the RID of SMRT or ACTR was expressed and purified from bacteria. Purified immobilized GST-SMRT(RID) or GST-ACTR(RID) fusion protein was incubated with *in vitro* transcribed and translated RARα, PML, or PML-RARα in the presence and absence of hormone. To our surprise, PML-RARα could be pulled down by GST-ACTR(RID) in both the presence and absence of hormone (Fig. 1A, lanes 4 and 5). No binding was observed with any of the proteins to GST alone. In contrast and as expected, RARα was able to bind to ACTR in a DNA-independent manner, but only after the addition of hormone (Fig. 1B, lane 4 versus lane 5). Also, PML was unable to bind to GST-ACTR(RID) regardless of the presence of hormone (Fig. 1C, lanes 4 and 5). As a control, Fig. 1D shows that the association of PML-RARα with SMRT in this assay is consistent with the well documented association, which requires 1 μM ATRA to induce complete dissociation (lanes 3–5). These results suggest that PML-RARα binds coactivators in solution in both the absence and presence of hormone.

To investigate the molecular basis of the hormone-independent association between PML-RARα and coactivators, we performed yeast-two hybrid assays. The yeast Gal4 activation domain (pGAD) was fused to the LBD of RARα, PML, or PML-RARα, whereas ACTR(RID) was fused to the Gal4 DNA-binding domain (pGBT9). The constructs were cotransformed into yeast. The interaction of ACTR(RID) and PML-RARα was assayed by measuring β-galactosidase expression. As shown in Fig. 2A, as controls, coexpression of the pGBT9 vector alone and pGAD-PML-RARα or pGBT9-ACTR(RID) and the pGAD vector alone resulted in only basal reporter activity. Moreover, coexpression of pGBT9-ACTR(RID) and pGAD-PML did not activate reporter activity in the presence or absence of hormone. As expected, coexpression of pGBT9-ACTR(RID) and pGAD-RARα(LBD) resulted in activation of the reporter only in the presence of hormone. However, consistent with our *in vitro* protein-protein interaction data (Fig. 1), coexpression of pGBT9-ACTR(RID) and pGAD-PML-RARα led to a potent activation of reporter activity in the absence of hormone, indicating a strong association between ACTR(RID) and PML-RARα. The addition of hormone modestly potentiated this interaction. Taken together, these results indicate that PML-RARα interacts with ACTR(RID) in yeast independently of hormone.

To test whether this interaction is unique only to ACTR, we performed a similar yeast two-hybrid analysis using constructs bearing the RID from SRC-1 and GRIP1. Fig. 2B demonstrates that, similar to ACTR(RID), RARα(LBD) associated only with SRC-1(RID) and GRIP1(RID) in the presence of hormone, whereas PML-RARα was able to associate with coactivators in both the presence and absence of hormone. To investigate whether this association extends to other histone acetyltransferases as well as the p160 family, yeast two-hybrid assays were performed to map the PML-RARα interaction domain within CBP (Fig. 2C). Our results show that, although coexpression of the pGAD vector and pGBT9-CBP(2–460) resulted in a slight activation of reporter activity (first bar), pGBT9-CBP(2–460) and pGAD-PML-RARα further activated expression of reporter activity (second bar), indicating an interaction between PML-RARα and CBP(2–460). The region of CBP involved in the interaction (residues 2–460) includes two LXXLL motifs at amino acids 68–78 and 355–365, which have previously been shown to be involved in associating with RARα (44). These data demonstrate that the hormone-independent interaction between PML-RARα and coactivators occurs with all three p160 coactivators tested as well as with CBP.

Because PML-RARα associated with the coactivators in a hormone-independent manner, a characteristic not shared with RARα, it is possible that the actual binding between PML-RARα and coactivators is distinct from that of RARα. To investigate this possibility, we performed yeast two-hybrid assays

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4 K. R. Yamamoto and A. So, personal communication.
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A

|               | no ligand | 1 μM AT-RA |
|---------------|-----------|------------|
| AD alone      | AD-RARα   | AD-PML-RARα| AD-PML-RARα |
| pGBT9 alone   | pGBT9-ACTR |

B

|               | none      | 1 μM AT-RA |
|---------------|-----------|------------|
| pGBT9-RAR     | pGBT9-PML-RAR | pGBT9-SRC-1| pGBT9-GRIP1 |
| pGBT9 alone   | pGBT9-ACTR |

C

|        | pGBT9 alone | pGBT9-PML-RARα |
|--------|-------------|----------------|
| 2-460  | 2-460       | 2-460          |
| 722-1162| 722-1162    | 722-1162       |
| 1602-1911| 1602-1911  | 1602-1911      |
| 1621-2342| 1621-2342  | 1621-2342      |

FIGURE 2. PML-RARα interacts with other coactivators in a hormone-independent manner. A, PML-RARα interacts with ACTR in yeast. Binding of PML-RARα to ACTR with or without 1 μM ATRA was assayed by yeast-two hybrid assays. Yeast expression plasmids pGBT9-ACTR and pGAD-PML-RARα were cotransformed into yeast strain Y190. Liquid β-galactosidase assays were performed according to the Clontech protocol. β-Galactosidase (β-gal) activity was normalized by total cell numbers and time. AD, activation domain. B, PML-RARα associates with other p160 family members in a hormone-independent manner. Binding of PML-RARα and RARα to SRC-1 and GRIP1 in the presence or absence of hormone was measured by yeast two-hybrid assays as described for A using pGBT9-SRC-1 (RID) or pGBT9-GRIP1 (RID) and either pGAD-RARα or pGAD-PML-RARα. C, PML-RARα associates with CBP in the absence of hormone. Binding of PML-RARα to CBP was assayed by yeast two-hybrid assays with pGAD-PML-RARα and different pGBT9-CBP constructs containing fragments of CBP as described for A.

using mutant ACTR constructs. There are three LXXLL-containing coactivator-binding motifs in ACTR(RID) (45). Each construct contained a single, double, or triple mutation of these three motifs, numbered 1–3 from the N terminus to C terminus. In the absence of ATRA, no interaction was observed (data not shown). In the presence of ATRA, we found that mutation of each of the motifs decreased the interaction with RARα. However, mutation of the second motif had the most severe effects on the association (Fig. 3A). The interaction was abolished in any of the constructs with a mutation of the second motif. A similar assay was performed for PML-RARα, except that yeast cells were grown in the absence of hormone. Fig. 3B shows that, similar to RARα, although all of the mutations decreased the association compared with the wild-type construct, mutations of the second motif abrogated the hormone-independent interaction. These data indicate that both the ligand-independent association of PML-RARα with ACTR and the ligand-dependent association of RARα with ACTR are mediated through LXXLL-containing motifs in the coactivators.

Although these data indicate that the hormone-independent association of coactivators with PML-RARα occurs in a manner similar to the hormone-dependent association with RARα, we hypothesized that there may also be contributions to this interaction due to the PML portion of PML-RARα, which is absent in RARα. To investigate this possibility, we generated PML-RARα constructs harboring a deletion of one functional region of the RBCC (ring finger/B boxes/coiled-coil domain) motif located in the N terminus of PML. These deletion constructs were tested for their ability to bind to PML-RARα in GST pulldown assay similar to that described for Fig. 1. In vitro translated PML-RARα proteins were incubated with immobilized GST-ACTR(RID) to compare the ability of the deletion mutants to associate with ACTR in the absence of hormone with that of wild-type PML-RARα. Interestingly, the deletion of PML regions did not seem to have any significant effect on the binding of PML-RARα to ACTR (Fig. 3C, lane 1 versus lanes 2–5). These data suggest that the LBD of the RARα portion of PML-RARα is the major contributor to the hormone-independent interactions with coactivators. To confirm this, we tested the binding of ACTR to a PML-RARα mutant containing a mutation at a site shown previously to abolish the interaction of RARα with coregulators in vivo by a mammalian two-hybrid assay (46). We found that F584A (corresponding to F249A in RARα) completely abolished the interaction of PML-RARα with ACTR in comparison with wild-type binding (lane 6).

To further analyze the activity of this mutant, we also confirmed by yeast two-hybrid analysis that the interaction of PML-RARα(F584A) was significantly less than that of wild-type PML-RARα with ACTR (Fig. 3D). These data show that, similar to the results from the yeast two-hybrid analysis of LXXLL motifs, the hormone-independent interaction between PML-RARα and coactivators appears to be mediated similarly to the hormone-dependent interaction between RARα and coactivators.

One main difference between PML-RARα and RARα is the ability of PML-RARα to form homodimers, a characteristic common to all fusion proteins expressed in APL. To test
whether this hormone-independent association with coactivators extends to other fusion proteins expressed in APL and therefore may be somewhat dependent on the homodimerization of the fusion protein, we tested the ability of PLZF-RARα/H9251 to associate with ACTR by GST pulldown assays. Similar to PML-RARα/H9251, PLZF-RARα/H9251 was capable of associating with ACTR in both the presence and absence of hormone (Fig. 3E). As a control, no interaction between PLZF and ACTR(RID) was observed under these conditions (Fig. 3F). Furthermore, this ligand-independent association was confirmed using a yeast-two hybrid assay (Fig. 3G). These data further support the idea that the hormone-independent association of PML-RARα with coactivators involves residues in the RARα portion of the fusion protein and is similar to that observed for the hormone-dependent association of RARα with coactivators.

To confirm that this hormone-independent association occurs in mammalian cells as well as in vitro, we performed co-immunoprecipitation studies. HA-PML-RARα was coexpressed with FLAG-ACTR in mammalian cells. Immunoprecipitations against the FLAG epitope and subsequent Western blotting with anti-FLAG and anti-HA antibodies were performed.
formed on whole cell lysates to examine whether ACTR forms a complex with PML-RARα (Fig. 4A). Our results show that HA-PML-RARα was detected in the immunopellet only when FLAG-ACTR was coexpressed, indicating an interaction between these two proteins. To determine whether this extends to other members of the coactivator complex, we performed a similar co-immunoprecipitation study with FLAG-PCAF coexpressed with either HA-PML-RARα or HA-RARα as a control (Fig. 4B). In this assay, immunoprecipitation was performed in both the presence and absence of ATRA. We found that, as expected, HA-RARα co-immunoprecipitated only with FLAG-PCAF in the presence of hormone (lanes 12 versus lane 14), whereas HA-PML-RARα was able to co-immunoprecipitate with FLAG-PCAF in both the absence and presence of hormone (lanes 11 and 13). To examine whether endogenous PML-RARα associates with coactivators, we prepared extracts from NB4 cells, which endogenously express PML-RARα. We examined whether coactivators are expressed in NB4 cells by immunoprecipitation followed by Western blotting. We found that the coactivators CBP, p300, and SRC-1 are expressed in NB4 cells (Fig. 4C). We then used an antibody against RARα to test whether it can precipitate PML-RARα. Fig. 4D demonstrates that this antibody was able to detect both RARα and PML-RARα (lanes 1–3 and data not shown) in these lysates. Subsequent stripping of this membrane and reprobing with anti-PML antibody confirmed that the higher molecular mass band detected with anti-RARα antibody is PML-RARα (lanes 4–6). To investigate whether the coactivators interact with PML-RARα, we performed co-immunoprecipitation using anti-coactivator antibodies for immunoprecipitation and anti-RARα antibody for Western blotting. Fig. 4E shows that all three coactivators were able to immunoprecipitate endogenous PML-RARα. Furthermore, we grew
NB4 cells in the presence of ATRA for 12 h and compared the ability of PML-RARα/H9251 and RARα/H9251 to immunoprecipitate with CBP in the absence and presence of hormone (Fig. 4F). As documented previously (47), PML-RARα/H9251 protein was decreased after ATRA treatment (lane 1 versus lane 2); however, anti-CBP antibody was able to efficiently immunoprecipitate PML-RARα under both conditions (lanes 3 and 4). Conversely, as expected, immunoprecipitation of RARα by CBP was readily detectable only in the presence of hormone (lanes 3 and 4). Taken together, these data demonstrate that PML-RARα associates with coactivators in mammalian cells in a hormone-independent manner.

To further characterize the interaction of PML-RARα with coactivators, we carried out immunofluorescence microscopy. To examine the colocalization of PML-RARα and coactivators in a cell line similar to cells endogenously expressing PML-RARα, we performed confocal microscopy with HL-60 cells (Fig. 5, A–C). In these cells, exogenous PML-RARα appeared to be localized mostly in the nucleus. Furthermore, PML-RARα partially colocalized with the
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cocativators SRC-1, CBP, and p300. However, because PML-RARα may not always localize to the nucleus in APL cells, we also tested the effects of PML-RARα on coactivators in CV-1 cells, in which PML-RARα is mostly cytoplasmic. We found that overexpression of PML-RARα in CV-1 cells led to a redistribution of coactivators. For SRC-1 and CBP, staining was mostly nuclear in untransfected cells, but more widespread and colocalized with PML-RARα in transfected cells (Fig. 5D, panels a–f). As a control, transfected RARα localized to the nucleus, and its overexpression in the cells was unable to disrupt the nuclear localization of coactivators (panels g–i). Furthermore, confocal microscopy studies confirmed these phenomena and indicated that, similar to SRC-1 and CBP, immunostaining for p300 showed a change from a mostly nuclear pattern in untransfected cells to a more diffuse pattern resembling that of PML-RARα in transfected cells (Fig. 5E), suggesting that PML-RARα colocalizes with p300. These data are consistent with our protein–protein interaction data, suggesting that PML-RARα interacts with coactivators in the absence of hormone in mammalian cells.

Although PML-RARα is capable of binding to DNA at a retinoic acid response element in vitro and possibly when overexpressed in vivo, there is currently no evidence that PML-RARα acts as a direct transcription factor in APL through direct regulation of RAR target genes. However, because PML-RARα is able to interact with coactivators in a hormone-independent manner, we hypothesized that PML-RARα could affect other genes whose activation involves binding to coactivators by possibly sequestering the coactivators from binding to other transcription factors. To test this hypothesis, we performed transient transfection assays. We first examined the effects of PML-RARα on Notch-mediated transactivation of the CBF1 transcription factor. TAN-1 is an intracellular fragment of the Notch transmembrane receptor that activates CBF1 activity through the recruitment of coactivator complexes, including PCAF and p300/CBP (48). To test whether PML-RARα has effects on the activation of CBF1, we carried out transient transfection experiments using a luciferase reporter gene construct containing a CBF1 response element (40). We found that, as expected, TAN-1 potently activated reporter activity (Fig. 6A, bar 2). Furthermore, TAN-1-mediated activation of CBF1 was attenuated by PML-RARα (Fig. 6B, bars 3–5); however, PML-RARα(F584A), which did not interact strongly with coactivators, did not have significant effects on reporter activity (bars 6–8). We also investigated whether PML-RARα has effects on activation of a luciferase reporter gene under the control of a GR response element. GR is a member of the family of class I nuclear receptors that bind DNA as homodimers to regulate transcription. In part, their activation of transcription is similar to RXRα/RARα in that they require hormone to bind a coactivator complex that includes the p160 coactivators and p300/CBP (49, 50). Fig. 6B demonstrates that, in the presence of dexamethasone, GR highly activated transcription of the GR response element-containing reporter construct (bars 1 and 2). The addition of PML-RARα led to decreased reporter activity in a dose-dependent manner (bars 3–6). Also, similar to TAN-1, this effect of PML-RARα was largely dependent on the association of PML-RARα with coactivators because PML-RARα(F584A) no longer had any significant effect on GR reporter activation (bars 7–10). Taken together, these results indicate that PML-RARα is able to inhibit the transcription activation of GR and CBF1 and that this inhibition depends on physical interaction with coactivators.

To further examine the effects of PML-RARα on GR transcription, we investigated the effects of exogenous PML-RARα on expression of endogenous GR target genes. GILZ and FLJ25390 are two genes that have been identified as GR targets in A549 cells and that are activated in response to dexamethasone. We analyzed these genes for transcription activity in the absence and presence of PML-RARα by RT-PCR. A549 cells were transfected with HA vector alone, HA-PML-RARα, or HA-PML-RARα(F584A). Cells were treated with dexamethasone, and the RNA was isolated for RT-PCR to measure expression of GILZ and FLJ25390. We found that PML-RARα, but not the vector alone or the F584A mutant, resulted in a decrease in activation of GILZ and FLJ25390 (Fig. 6C, lanes 3 and 4 versus lanes 1, 2, 5, and 6). Quantification analysis indicated that expression of PML-RARα blocked the hormone-induced expression of GILZ and FLJ25390, whereas expression of the vector alone and PML-RARα(F584A) did not have this effect. Furthermore, GILZ was expressed and regulated by dexamethasone in NB4 cells, an APL patient-derived cell line that expresses PML-RARα. To test whether PML-RARα affects GILZ gene expression in these cells, we treated the cells with a concentration of ATRA shown previously to induce degradation of PML-RARα (47). RT-PCR analysis of GILZ indicated that dexamethasone treatment resulted in an increase in GILZ expression. In addition, when ATRA was also included with dexamethasone, there was a further increase in GILZ expression (Fig. 6D). These results indicate that the presence of PML-RARα, even at endogenous levels, results in a decrease in GR target gene expression. These data are consistent with our model in that, by interacting with coactivators in a hormone-independent manner, PML-RARα is able to sequester these transcription regulators from associating with transcription factors that rely on them for proper control of transcription.

DISCUSSION

To further define the mechanism of transcription regulation by PML-RARα, we undertook a study of the association of PML-RARα with coactivators. PML-RARα is known to have an aberrant hormone responsiveness that results in reduced dissociation of corepressors. To our surprise, we found that unliganded PML-RARα interacted with coactivators both in mammalian cells and in vitro. This result is reminiscent of our previous study in which RXRα/RARαΔ443, a helix 12 deletion mutant, acquired not only hormone-resistant dissociation with the corepressors, but also hormone-independent association with the coactivators (46). In addition, hormone further promoted the association between PML-RARα and coactivators, as observed in GST pulldown assays (Fig. 1) and electrophoretic mobility shift assays (data not shown). In the context of PML-RARα being a constitutive repressor,
this hormone-promoted coactivator binding likely plays a role in the response of patients to pharmacological levels of ATRA by mediating the release of corepressors and the binding of coactivators to PML-RARα.

Through the use of our mutation and deletion analysis of PML-RARα to investigate this interaction, we have shown that the region of binding for the hormone-independent association is similar to that for hormone-dependent interactions of PML-

FIGURE 6. PML-RARα abrogates transcription activation by GR and CBF1. A, PML-RARα inhibits transcription activation by CBF1. Increasing concentrations of PML-RARα were cotransfected with TAN-1. The transcription activation by TAN-1 of a consensus CBF1 response element was assayed. WT, wild-type. B, PML-RARα antagonizes hormone-dependent activation by GR. Increasing concentrations of PML-RARα were cotransfected with GR in cells treated with 1 μM dexamethasone. The transcription activation by GR of a consensus GR response element was assayed. Transient transfection experiments were carried out as described under “Experimental Procedures.” C, PML-RARα overexpression results in decreased activation of endogenous GR target genes. RT-PCR was performed to analyze the transcription levels of GILZ and FLJ25390 on RNA isolated from A549 cells transfected with the HA vector, HA-PML-RARα, or HA-PML-RARα(FS84A) and either untreated or treated with 1 μM dexamethasone (Dex). Whole cell lysates were analyzed for expression of wild-type HA-PML-RARα and HA-PML-RARα(FS84A) by Western blotting with anti-HA or anti-green fluorescent protein (GFP) antibody as a transfection control. Quantification of the intensity of the RT-PCR products is shown. Bar 1, HA; bar 2, HA-PML-RARα; bar 3, HA-PML-RARα(FS84A). The graphs represent the amount of product obtained from dexamethasone-treated samples over those that were untreated. All bands are relative to the actin levels of each sample as a control. D, treatment of ATRA results in increased activation of an endogenous GR target gene in NB4 cells. RT-PCR was performed on RNA isolated from NB4 cells after treatment with ATRA and/or dexamethasone to analyze expression of GILZ. Quantification of the intensity of RT-PCR products is shown and was analyzed as described for C.
RARα and RARα with coactivators. Although deletion of the functional domains of PML in PML-RARα had no drastic effect on the ability to bind to ACTR, the loss of the RING domain did appear to decrease the interaction to ~60% that of the wild-type protein; however, how the function of the RING domain may affect this interaction is currently unclear. Furthermore, a mutation in the LBD of RARα, found previously to abolish the interactions with coregulators (46), had a similar effect on PML-RARα, indicating that the LBD is primarily responsible for this interaction. On the basis of these results, we suggest that, although the main site of interaction between PML-RARα and coactivators in the absence of hormone is the LBD of RARα, it is likely that the PML portion of the fusion protein may have some effects on this interaction due to dimerization status and interactions with other proteins in the cell. This hormone-independent association with coactivators is not unique to PML-RARα among APL fusion proteins, as we also observed the association to occur with PLZF-RARα. Likewise, a similar hormone-independent interaction between NPM-RARα and ACTR has been observed previously (38). Whether or not this interaction plays a role for all of the different APL fusion proteins remains to be tested, however, because recent evidence suggests that the different fusion partners of RARα result in leukemias with different biological features in a mouse model of APL (51).

Although the structural basis for the interactions between coregulators and PML-RARα is currently undefined, our and other results validate a mechanistic link of the interactions between receptors and the coactivators and corepressors. These studies with both corepressors and coactivators further suggest that, although the LBDs of the fusion proteins are similar to that of wild-type RARα, it is likely that there are small conformational changes that are responsible for the differential binding to these coregulators. We propose that the association of PML-RARα with coactivators results in sequestration of these proteins, limiting their availability to bind other transcription factors. This would then result in aberrant transcription of these genes, as indicated by our studies with a reporter gene under the control of either GR or CBFI, whose transcription activation requires association with the coactivators, including GRIP1 and/or p300/CBP. This hypothesis is further supported by our data indicating that ectopic expression of PML-RARα in A549 cells results in a decrease in dexamethasone-induced activation of endogenous GR target genes GILZ and FLJ25390 (Fig. 6C). Although the effects of this overexpression may seem modest at only an ~20–30% reduction in the amount of transcription induced by dexamethasone for PML-RARα compared with that for either the vector alone or the F584A mutant, which has a reduced affinity for coactivators, it is important to note that the RT-PCR was performed on the whole population of cells, not just those transfected. Furthermore, our data in NB4 cells analyzing GILZ expression before and after treatment with ATRA to induce degradation of PML-RARα indicate that this effect is not only a result of PML-RARα overexpression and therefore may play a role in APL leukemia. The effects of PML-RARα on target genes are likely dependent on the environment and the local concentration of PML-RARα around the promoter, as we did not observe an effect of PML-RARα on the transcription activation of either estrogen receptor-α or MEF2 (supplemental Fig. 1). We hypothesize that the binding affinity and preference of PML-RARα and the target transcription factor for different coactivators play a role in determining the level to which PML-RARα may affect its transcription activation.

Our model, in which PML-RARα has effects on a wide array of other cellular targets, supports a previous report demonstrating that PML-RARα affects both nuclear receptor as well as non-nuclear receptor signaling in myeloid differentiation (8). Coincidentally, recent microarray data on genes whose expression changed upon retinoic acid treatment of APL blasts or U937 cells expressing either wild-type RARα or PML-RARα reveal that a number of genes show similar patterns of expression in the presence of the two receptors. Furthermore, there are subsets of genes unique to each (52, 53), indicating that PML-RARα also has effects on expression of non-RARα target genes and further suggesting that PML-RARα does not simply bind and/or affect only genes regulated by RARα.

Determining the exact endogenous localization of PML-RARα is difficult at this point due to the lack of an appropriate cell line and the lack of appropriate antibodies that specifically recognize PML-RARα without cross-reacting with PML or RARα. Two of the cell lines employed in this study show differential subcellular distribution. In HL-60 cells, we found a mostly nuclear localization of overexpressed PML-RARα, though it was unevenly distributed throughout the nucleus. However, because myoblasts, which express PML-RARα, have large nuclei and very little cytoplasm, it is difficult to visualize changes in protein subcellular localization. To avoid this problem and to further support our data, we transiently overexpressed HA-PML-RARα in CV-1 cells, a cell line used throughout the nuclear receptor field and in which movement of proteins between the nucleus and cytoplasm is more easily detected, and performed confocal microscopy to examine any effect overexpressed HA-PML-RARα had on the endogenous coactivators. We found that PML-RARα is localized predominantly in the cytoplasm in this cell line. In either case, a significant amount of colocalization of PML-RARα and coactivators was observed, indicating possible interaction in the absence of hormone. Notably, we have demonstrated that endogenous PML-RARα associates with coactivators in NB4 cells. We hypothesize that the hormone-independent association with coactivators likely plays a role in situations in which PML-RARα may not be completely nuclear, as was reported for APL blasts (54, 55), as well as when PML-RARα is likely nuclear due to overexpression of PML-RARα in the cell, enabling it to sequester a large portion of the coregulators (8, 56).

Nevertheless, the unique properties of PML-RARα based on our findings will give rise to many questions in redefining its mechanism of action. First, because the binding of corepressors and coactivators to PML-RARα is mutually exclusive and because both species exist in at least some physiological settings, there must be tight regulation of the interplay between these factors in the absence or presence of low levels of hormone. It has been suggested that PML-RARα may act in some circumstances to decrease the physiological concentration of corepressors (34, 35). This would then alter the ratio of core-
pressors to coactivators, and based on our findings, it may lead to alternate transcription regulation by PML-RARα. Second, it is likely that PML-RARα binding of corepressors or possible sequestering of coactivators preferentially affects transcription of some genes over others. In accordance with this idea, it has been suggested that, because PML-RARα disrupts the normal PML localization in PML bodies, it may relocalize cofactors by subsequently disrupting their normal localization, thereby preventing them from functioning normally (57). Third, it is possible that PML-RARα interacts with other transcription factors to modulate transcription by recruiting coactivators or corepressors, similar to one mechanism by which GR has been proposed to affect transcription (49, 50). Understanding the role of PML-RARα and deciphering its interactions with coregulators under these different circumstances will help to further determine the altered signaling that leads to the differentiation block.

The current treatments for APL, which involve targeting PML-RARα for degradation and inhibiting the activity of histone deacylases, can unfortunately have various deleterious effects in the cell due to interference with the wild-type PML and corepressors, similar to one mechanism by which GR has been proposed to affect transcription (49, 50). Recent suggestions for treatment also include targeting other oncogenic pathways that may be involved in APL such as the Ras pathway (61), but these would be in addition to the traditional treatments. In the same token, long-term cancer management with the available treatments, the most common being pharmacological levels of ATRA, can lead to hormonal resistance, which impedes the ability of the drugs to help the patient overcome the illness. Finally, although most APL patients express fusions with PML, which are ATRA-responsive, there are forms of PML-RARα that allow coregulator associations. This may present possible targets of therapies that could more specifically act on the fusion protein to block its action while leaving other cellular functions intact, thus decreasing side effects and hopefully increasing the potency of treatment and widening the available patient base to better treat all APL patients. Ground work on targeting transcription factor-coregulator associations has been started, and it has been shown that a small peptide can be used to induce dissociation of the corepressor SMRT from Bcl-6 (62, 63), suggesting that, with more knowledge of these properties of PML-RARα, new and improved possibilities for patient treatment could be on the horizon.

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