Inhibition of the Peptidyl-Prolyl-Isomerase Pin1 Enhances the Responses of Acute Myeloid Leukemia Cells to Retinoic Acid via Stabilization of RARα and PML-RARα

Maurizio Gianni',[1] Andrea Boldetti,[1] Valeria Guarnaccia,[1] Alessandro Rambaldi,[2] Edoardo Parrella,[1] Ivan Raska, Jr.,[1] Cecile Rochette-Egly,[3] Giannino Del Sal,[4] Alessandra Rustighi,[4] Mineko Terao,[1] and Enrico Garattini[1]

[1]Laboratory of Molecular Biology, Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy; [2]Division of Hematology, Ospedali Riuniti di Bergamo, Bergamo, Italy; [3]Department of Functional Genomics, Institut de Genetique et Biologie Moleculaire, Illkirch, France; and [4]Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, Università di Trieste; Dipartimento di Biochimica Biofisica e Chimica delle Macromolecole, Trieste, Italy

Abstract

The peptidyl-prolyl-isomerase Pin1 interacts with phosphorylated proteins, altering their conformation. The retinoic acid receptor RARα and the acute-promyelocytic-leukemia–specific counterpart PML-RARα directly interact with Pin1. Overexpression of Pin1 inhibits ligand-dependent activation of RARα and PML-RARα. Inhibition is relieved by Pin1-targeted short interfering RNAs and by pharmacologic inhibition of the catalytic activity of the protein. Mutants of Pin1 catalytically inactive or defective for client-protein–binding activity are incapable of inhibiting ligand-dependent RARα transcriptional activity. Functional inhibition of RARα and PML-RARα by Pin1 correlates with degradation of the nuclear receptors via the proteasome-dependent pathway. In the acute myelogenous leukemia cell lines HL-60 and NB4, Pin1 interacts with RARα in a constitutive fashion. Suppression of Pin1 by a specific short hairpin RNA in HL-60 or NB4 cells stabilizes RARα and PML-RARα, resulting in increased sensitivity to the cytodifferentiating and antiproliferative activities of *all-trans* retinoic acid. Treatment of the two cell lines and freshly isolated acute myelogenous leukemia blasts (M1 to M4) with ATRA and a pharmacologic inhibitor of Pin1 causes similar effects. Our results add a further layer of complexity to the regulation of nuclear retinoic acid receptors and suggest that Pin1 represents an important target for strategies aimed at increasing the therapeutic index of retinoids. [Cancer Res 2009;69(3):1016–26]

Introduction

All-trans retinoic acid (ATRA) is a physiologic modulator of myeloid cells. Most of the activities of ATRA and derivatives (retinoids) are mediated by specific nuclear receptors, which are ligand-dependent transcription regulators acting as retinoid X receptor/retinoic acid receptor (RXR/RAR) heterodimers (1). The receptors control target genes via binding to cognate DNA consensus sequences [retinoic acid responsive element (RARE)].

ATRA is the only differentiating agent used in the clinics, being part of the standard treatment of acute promyelocytic leukemia (APL; ref. 2). The majority of APL cases is characterized by expression of PML-RARα (3, 4), an aberrant form of RARα, which is the major retinoid receptor in myeloid cells. ATRA induces maturation of the APL blast along the granulocytic pathway (4). Despite expression of RARα (5), acute myelogenous leukemia (AML) types other than APL are generally refractory to retinoid-induced differentiation. This and toxicity issues hamper a more general use of ATRA and derivatives in the management of AML. Strategies aimed at increasing the therapeutic index of retinoids are important goals (6–11).

The activity and stability of nuclear retinoid receptors, including pathologic PML-RARα, and accessory proteins are modulated by various signals, such as phosphorylation events (12–14). The proteolytic degradation of RARs, PML-RARα, coactivator, and corepressors via the proteasome-dependent pathway is an emerging control system for the activity of retinoid receptor complexes (15–17). The regulation of these multilayered processes offers many opportunities of pharmacologic intervention aimed at potentiating the therapeutic activity of ATRA (18).

Pin1 is a unique peptidyl-prolyl-isomerase recognizing phosphorylated Ser(Thr)-Pro motifs of client proteins (19). Pin1 isomerizes the Ser(Thr)-Pro bond from *cis* to *trans*, altering the conformation of target proteins (20), often routing them along the proteasome-dependent degradation pathway (19, 21, 22). The enzyme is an interesting target of intervention, as it is overexpressed in various types of neoplasia (23), including AML (24), and RARα may be one of its client proteins (25).

Materials and Methods

Cells. NB4 (26), HL-60 (27), NB4.007 (28), HL-60R (29), PR-9 (30), COS-7 cells, and freshly isolated AML blasts from the peripheral blood of five patients (Supplementary Table S1) were cultured as described (6, 7, 9, 11, 31). To generate polyclonal populations of NB4 and HL-60 cells silenced for Pin1, the two cell lines were electroporated with a plasmid containing a short hairpin RNA (shRNA) targeting the peptidyl-prolyl-isomerase (32).

Reagents. Diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[*lmn*][3,8]phenanthroline-2,7-diacetate (PiB), MG132, and ATRA were from Calbiochem and Sigma. The predesigned Pin1 (S10545)–targeted and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–targeted short interfering RNAs (siRNA) were obtained from Ambion.

Pull-down, far-Western, immunoprecipitation, and Western blot analyses. Pull-down experiments (33) were conducted on COS-7 cells transiently transfected with RARα and PML-RARα cDNAs (11). Proteins were precipitated with a purified glutathione *S*-transferase (GST)

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Enrico Garattini, Istituto di Ricerche Farmacologiche "Mario Negri," via La Masa 19, 20156 Milan, Italy. Phone: 39-02-39014533; Fax: 39-02-3546277; E-mail: egarattini@marionegri.it.

doi:10.1158/0008-5472.CAN-08-2603

Pin1 fusion protein after incubation with glutathione-Sepharose beads (Amersham). Precipitated proteins were subjected to Western blot analysis using anti-RARα or anti-GST antibodies (Santa Cruz Biotechnology). Pull-down experiments were also done with a GST-fusion protein of the Pin1 WW domain (33) and purified recombinant RARα (34). For these experiments, RARα was preincubated with catalytically active extracellular signal-regulated kinase-1 (ERK1; Cell Signaling Technology).

Far-Western experiments (35) were performed on COS-7 cell extracts transfected with a RARα cDNA. Cell extracts were immunoprecipitated with anti-RARα antibodies [Mab 9α (F)] (14), and incubated with purified recombinant GST-tagged *wild-type* (*WT*), *WW-domain, Y23/A*, and *C113/A* mutants of Pin1 (32, 33). Blots were developed with the anti-Pin1 (Millipore) or anti-RARα (36) antibodies.

Immunoprecipitation (anti-RARα and anti-Pin1 antibodies) and Western blot experiments were performed on cellular extracts of HL-60, NB4, or COS-7 cells transfected with Pin1 and RARα cDNAs (17). Other Western blots were developed with anti-actin, anti-cEBPβ, anti–STAT-1, anti–phospho-STAT-1, and anti-RXRα (11, 16) antibodies (Santa Cruz Biotechnology).

***RARα* and *PML-RARα* transactivation.** COS-7 cells were transfected with RARα, PML-RARα, and/or WT as well as mutant Pin1 cDNAs (*WW-domain, Y23/A*, and *S67/E*, which is catalytically inactive and functionally equivalent to *C113/A*; refs. 32, 33) in the presence of the RARE-containing DR5-tk-CAT (chloramphenicol-acetyl transferase; ref. 9), DR5-tk-luciferase or β2RARE-luciferase reporter constructs (37). For the luciferase and CAT reporter genes, the normalization plasmids are a renilla luciferase construct (Promega) and pCH110 (containing the bacterial β-galactosidase cDNA; ref. 9), respectively. CAT (9), firefly, and renilla luciferase (Promega) as well as β-galactosidase (9) activities were measured as detailed. The transactivation experiments conducted in NB4 and HL-60 cells as well as derived shRNA stably transfected cells were performed with an electroporation apparatus (Bio-Rad) using a described protocol (37).

Fluorescence-activated cell sorting, nitroblue tetrazolium reductase, and real-time reverse transcription-PCR. Fluorescence-activated cell sorting (FACS) analysis and determination of nitroblue tetrazolium reductase (NBT-R) activity were performed as described (11). Real-time reverse transcription-PCR (RT-PCR) was performed with Taqman gene expression assays (RARβ, Hs00233407_m1; Egr1, Hs00152928_m1; CYP26, Hs00175627_m1; β-actin endogenous control, 4333762F; Applied Biosystems). Amplification of the paxillin (PXN) mRNA was performed in the presence of the Sybr-green dye (Applied Biosystems). In the case of RARβ, the results are expressed as relative quantities of the amplified mRNA, using β-actin as the external amplification standard. The number of cycles necessary for the amplification of β-actin does not vary significantly across the various experimental points (15–16 cycles). The amount of RARβ transcript amplified in *pR-NB4* and *pR-HL-60* cells after treatment with ATRA is considered to have a value of 1; "nd" means the value is below the limit of detection of the assay (lack of signal after 35 cycles of amplification). In the case of all the other transcripts, the values are expressed as the ratio of the QR values [QR = $2^{-\Delta\Delta Ct}$; Ct = cycle threshold; ΔCt = Ct of the test mRNA − Ct of β-actin; ΔΔCt = ΔCt − ΔCt of the calibrator (vehicle-treated sample)] observed in *pR-PIN-NB4* or *pR-PIN-HL-60* and the appropriate *pR-NB4* or *pR-HL-60* controls. The following amplimers were used for the amplification of PXN: 5′-CATGTACGTCCCCACGAACTG-3′ (nucleotide 2025–2045 of the PXN cDNA); 5′-CACTGCTGAAATATGAGGAAGAGATG-3′ (complementary to nucleotide 2072–2095 of the PXN cDNA). The sequence of the β-actin mRNA amplimers is as published (38).

Pin1 silencing in NB4 and HL-60 cells. To generate polyclonal populations of NB4 and HL-60 cells silenced for Pin1, the two cell lines were electroporated with a plasmid construct containing a short hairpin RNA (shRNA) targeting the peptidyl-prolyl-isomerase (32). All the comparisons were made with the same cell lines transfected with void plasmids. However, comparative experiments on the ATRA-dependent expression of several myeloid markers were also performed in NB4 cells transfected with an irrelevant shRNA (targeting β-glactosidase) to rule out off-target effects of the Pin1 targeting shRNA (data not shown and Fig. 4*A*). Cell populations with stable expression of the shRNA or control plasmid were selected in RPMI 1640 complete medium containing 50 μg/mL puromycin (Sigma).

Results

Direct and ligand-independent interaction of Pin1 with RARα or PML-RARα. To study the interaction of Pin1 with RARα and PML-RARα, COS-7 cells were transfected with the corresponding cDNAs and treated with either vehicle or ATRA (1 μmol/L) for 2 hours. Pull-down experiments were performed with a GST-Pin1 fusion protein or a GST negative control (Fig. 1*A*). RARα and PML-RARα bound to Pin1 in the absence of ATRA. The binding did not seem to be significantly influenced by the retinoid. To confirm the interaction between RARα and Pin1, immunoprecipitation experiments coupled to Western blot analyses with anti-Pin1 and anti-RARα antibodies were performed (Fig. 1*B*). In cells forced to express Pin1, similar amounts of the peptidyl-prolyl-isomerase were recovered in anti-Pin1 immunoprecipitates obtained after treatment with vehicle and ATRA, whereas small amounts of endogenous RARα were coprecipitated in either condition and were visible only upon extended exposure of the blot. Significant amounts of RARα were complexed to Pin1 in cells cotransfected with RARα and Pin1 cDNAs. Treatment of cells with ATRA had no significant effect on the amount of Pin1 bound to RARα. Ligand-independent complexing of RARα to Pin1 was confirmed by mirror experiments involving immunoprecipitation with an anti-RARα antibody.

Far-Western experiments indicated that the interaction between Pin1 and RARα is direct and ligand independent (Fig. 1*C*). Indeed, a specific band corresponding to the receptor was recognized by GST-Pin1 after gel electrophoresis of COS-7 cell extracts transfected with the RARα cDNA, regardless of ATRA treatment. Pin1 binds to client proteins via a fundamental tyrosine residue (Y23) located within the WW amino-terminal domain (22, 33). Furthermore, binding does not require a catalytically active carboxyl-terminal domain. As expected, substitution of Y23 with an alanine (*Y23/A*) suppressed the ability of Pin1 to interact with RARα. In contrast, a truncated version of Pin1 corresponding to the entire WW domain or a catalytically inactive version of the protein (*C113/A*) maintained the ability to interact with RARα. Further support for a direct interaction was provided by the observation that the GST-Pin1 WW domain bound to *in vitro* translated RARα. Binding required phosphorylation of RARα (39), afforded by preincubation with recombinant ERK1, which phosphorylates the same consensus sequences recognized by Pin1 (40).

The ligand-independent interaction between RARα or PML-RARα and Pin1 was substantiated in the retinoid-sensitive AML-derived HL-60 and APL-derived NB4 cell lines (Fig. 1*D*). Similar levels of RARα could be immunoprecipitated by anti-Pin1 antibodies from extracts of HL-60 cells treated with vehicle or ATRA for 2 hours. By the same token, equivalent amounts of Pin1 were immunoprecipitated with anti-RARα antibodies regardless of the presence of ATRA in the culture medium at both 2 and 24 hours. In NB4 cells, not only RARα but also PML-RARα was coprecipitated by anti-Pin1 antibodies. Even in this cell line, the interaction between RARα or PML-RARα and Pin1 was supported by mirror experiments performed with the anti-RARα antibodies. As observed in the case of HL-60 blasts, treatment of NB4 cells with ATRA did not result in significant changes in the ability of Pin1 to interact with either retinoic acid receptor. Interestingly, Pin1 was identified in the transcriptional complexes bound to functional RAREs of the two direct retinoid-responsive genes, *CD38* (Supplementary Fig. S1) and *RARβ* (data not shown), both in the absence and presence of ATRA. This was assessed by chromatin immunoprecipitation assays performed with anti-Pin1 and

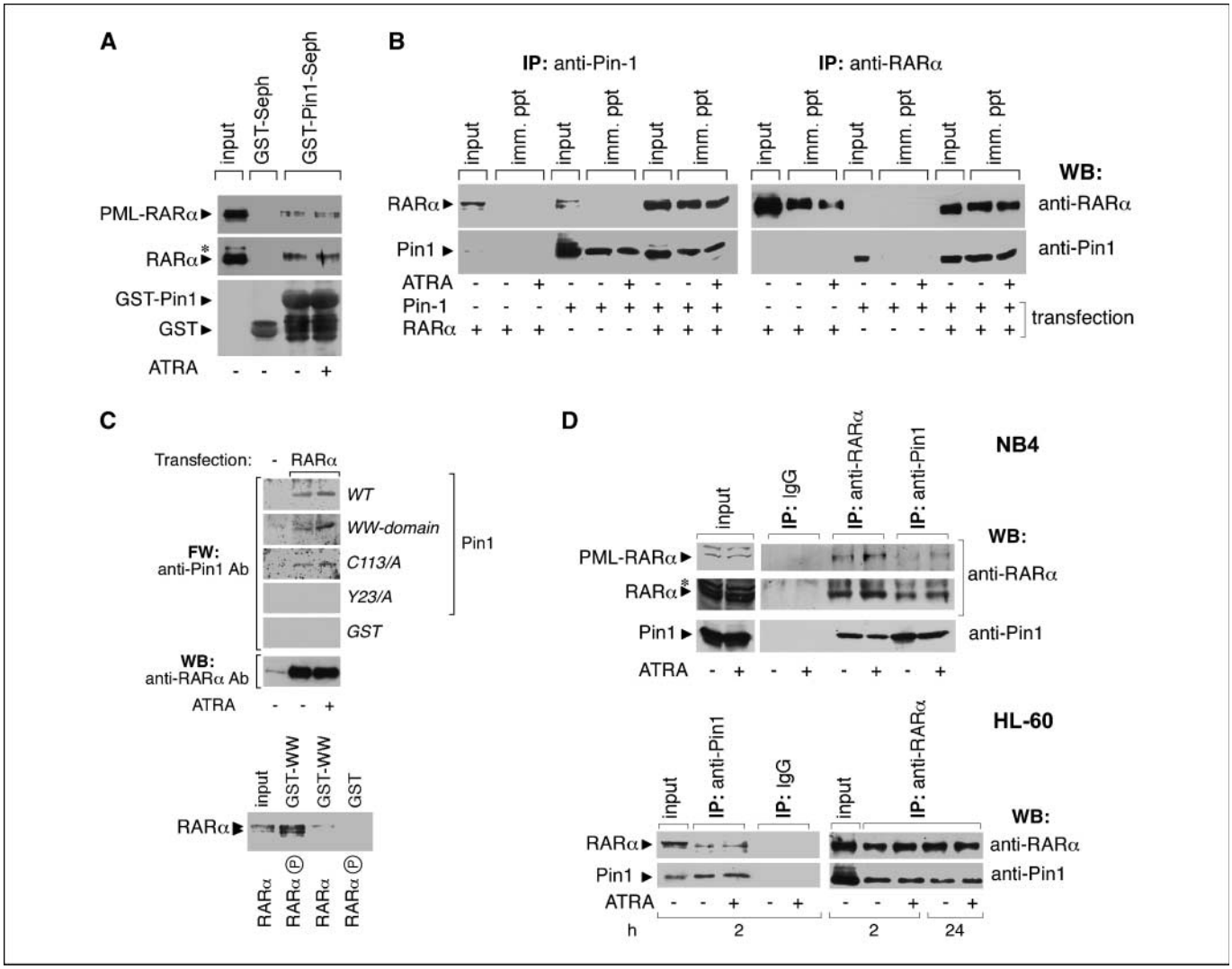

Figure 1. Interaction between Pin1 and RARα or PML-RARα. *A,* COS-7 cells were transfected with RARα (0.1 μg) or PML-RARα (0.1 μg) and treated with vehicle (DMSO) or ATRA (1 μmol/L) for 2 h. Cell extracts were pulled down with GST-Pin1 immobilized on Sepharose beads or the GST-negative control. Precipitated proteins were subjected to Western blot analysis with anti-RARα (*top two panels*) and anti-GST tag (*bottom panel*) antibodies. The input of PML-RARα and RARα present in transfected COS-7 cells is shown on the left. This corresponds to 1/20th of the material used for the pull-down. *B,* COS-7 cells were transfected with RARα (0.1 μg) and/or Pin1 (0.4 μg). After treatment for 2 h with ATRA (1 μmol/L), cell extracts were immunoprecipitated with anti-Pin1 (*IP, left*) or anti-RARα (*IP, right*) antibodies. The viability of COS-7 cells was >90% in all the experimental conditions. Immunoprecipitates were subjected to Western blot analysis (*WB*) with anti-RARα and anti-Pin1 antibodies. *C, top panels,* Far-Western experiments (*FW*) were conducted on extracts of COS-7 cells transfected with RARα (0.5 μg) treated with vehicle or ATRA (1 μmol/L) for 2 h. Blots of the extracts were incubated with recombinant *WT, WW-domain, Y23/A*, and *C113/A* mutant Pin1 proteins and developed with anti-Pin1 antibodies. The bottom lanes illustrate the levels of RARα expression, as assessed by Western blotting. *Bottom panel,* a recombinant RARα protein was incubated for 20 min in the absence (*RARα*) or in the presence of recombinant ERK1 to phosphorylate the protein (*RARα P*). An aliquot of the incubation mixture was pulled down with a GST-tagged version of the WW domain of Pin1 (*GST-WW*) immobilized on Sepharose beads or the GST-negative control. Precipitated proteins were subjected to Western blot analysis with anti-RARα antibodies. One tenth of the RARα input is shown on the left. *D,* HL-60 and NB4 cells treated with DMSO or ATRA (1 μmol/L) for 2 and/or 24 h. The viability of NB4 and HL-60 cells was >90% in all the experimental conditions. Cell extracts were immunoprecipitated (*IP*) with anti-Pin1 and anti-RARα antibodies or the IgG-negative control. The immunoprecipitates were subjected to Western blotting with anti-Pin1 or anti-RARα antibodies. In the case of HL-60, input RARα is not different in extracts of vehicle-treated and ATRA-treated (data not shown) cells. The asterisks indicate a nonspecific band recognized by the anti-RARα antibody.

anti-RARα antibodies on the retinoid-responsive regulatory region of the AML-specific *CD38* gene in HL-60 cells.

Pin1 inhibits ligand-dependent transcriptional activation of RARα or PML-RARα; silencing or pharmacologic inhibition of Pin1 suppresses the effect. The functional consequences of the interaction between Pin1 and RARα or PML-RARα were studied in COS-7 cells (Fig. 2*A*). Cotransfection of the Pin1 and RARα cDNAs resulted in dose- and time-dependent inhibition of the receptor transactivation. With 0.4 μmol/L Pin1, inhibition was evident up to 48 hours (data not shown). Similar results were observed if the RARα cDNA was substituted for by the PML-RARα counterpart. The experiment shown was performed with a luciferase reporter gene controlled by artificial RAREs (*DR5-TK-Luc*). Equivalent results were routinely obtained with a similar construct in which the luciferase was substituted by a chloramphenicol-acetyl-transferase (CAT) reporter (see also Fig. 2*B*). Inhibition of ATRA-dependent activation of RARα by Pin1 was also observed with luciferase-based constructs containing the natural retinoid-responsive promoter of human *RARβ2* (ref. 41; data not shown). The specificity of the inhibitory effect was verified using two siRNAs targeting different regions of

the Pin1 transcript and one siRNA targeting *GAPDH* as a negative control (Fig. 2*B*). Consistent with specific silencing (only siRNA A is shown; *bottom*), both Pin1-targeted siRNAs, but not the GAPDH counterpart, suppressed the inhibitory effect of Pin1 on the transcriptional activity of RARα. Noticeably, cotransfection of RXRα did not alter the inhibitory effect afforded by Pin1 on the ligand-dependent transactivation of RARα (Supplementary Fig. S2*A*).

Pin1-dependent inhibition of RARα activity requires the presence of a functionally competent peptidyl-prolyl-isomerase (Fig. 2*C*). In line with the observed inability of Pin1*Y23/A* to interact with the receptor (Fig. 1*C*), transfection of COS-7 cells with the mutant failed to inhibit ligand-dependent transactivation of RARα. The prolyl-isomerase defective *S67/E* mutant, which is functionally equivalent to *C113/A*, was equally incapable of

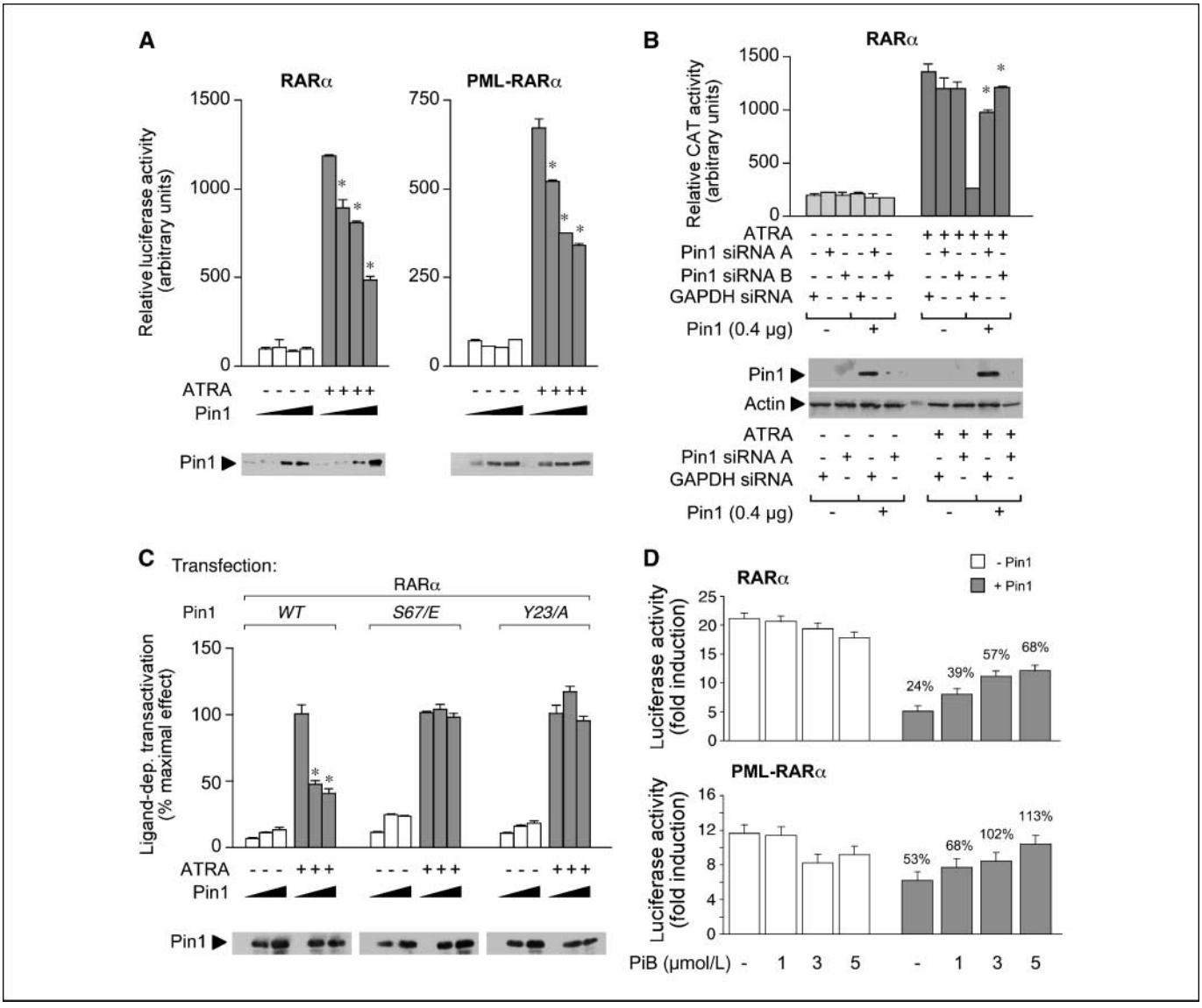

Figure 2. Consequences of Pin1 overexpression on RARα or PML-RARα transcriptional activity: effects of Pin1 silencing, pharmacologic inhibition, and functional inactivation. *A,* COS-7 cells were transfected with RARα or PML-RARα (0.1 μg) in the presence of Pin1 (0.1, 0.2, and 0.4 μg), a RARE-tk-Luc reporter (1 μg), and the renilla luciferase construct (0.5 μg). Twenty-four hours after transfection, cells were treated with DMSO or ATRA (1 μmol/L) for a further 24 h. The activity of firefly luciferase was measured and normalized for transfection efficiency using renilla luciferase activity. *Columns,* mean of duplicate transfections; *bars,* SD. The lower Western blots document the level of Pin1 in the cell extracts used for the transactivation assays. *, significantly lower than the relative ATRA-treated control (Student's *t* test, $P < 0.01$). *B,* COS-7 cells were transfected with the RARα cDNA (0.1 μg), the RARE-tk-CAT reporter (1 μg), and the pCH110 normalization plasmid (0.5 μg), in the presence or absence of the Pin1 cDNA (0.4 μg). Transfection mixtures also contained the indicated siRNAs (20 nmol/L). Cells were treated as in *A*. CAT activity was measured in cell extracts and normalized with bacterial β-galactosidase (*top*). *Columns,* mean of duplicate transfections; *bars,* SD. Western blots of Pin1 and actin are also shown (*bottom*). *C,* COS-7 cells were transfected with RARα (0.1 μg) in the presence of Pin1 or the indicated mutants (0.1, 0.2, and 0.4 μg), the RARE-tk-Luc reporter, and renilla luciferase (0.5 μg), then treated and processed for the measurement of luciferase activity as in *A. Columns,* mean of duplicate transfections; *bars,* SD. Western blots of Pin1 are also shown (*bottom*). *D,* COS-7 cells were transfected with RARα (0.1 μg), a RARE-tk-Luc reporter (1 μg), and the renilla luciferase normalization plasmid (0.5 μg), in the presence or absence of the Pin1 cDNA (0.4 μg). Cells were treated with DMSO or ATRA (1 μmol/L) in the presence or absence of the indicated concentration of PiB for 24 h as in *A*. Cell viability is always ≥90% in all the experimental conditions. The amount of luciferase activity was measured as in *A*. Results are expressed in fold induction, which is the ratio of the luciferase activity measured in extracts of cells treated with ATRA and the corresponding DMSO control. The values above the columns indicate the percentage of luciferase activity determined in RARα+Pin1 relative to RARα transfected cells. *Columns,* mean of duplicate transfections; *bars,* SD.

inhibiting ATRA-dependent activation of RARα. Interestingly, pharmacologic inhibition of Pin1 with PiB (42) was sufficient to cause dose-dependent reversion of the inhibitory effect afforded by Pin1 overexpression (Fig. 2*D*).

Pin1 induces ligand-independent degradation of RARα and PML-RARα by the proteasome pathway. To get insights into the mechanisms underlying the inhibitory effect on RARα and PML-RARα, we compared the levels of the two proteins in COS-7 cells transfected with Pin1 (Fig. 3*A*). In the absence of ATRA, Pin1 caused a dose-dependent decrease in the steady-state levels of both RARα and PML-RARα. As expected, treatment of cells with ATRA induced degradation and, consequently, a net decrease of the steady-state levels of the two receptors (15, 43). As ATRA-dependent degradation of RARα and PML-RARα was already maximal, overexpression of Pin1 did not cause any additional effect. Silencing of Pin1 with a specific siRNA increased the basal levels of RARα observed in COS-7 cells transfected with Pin1 (Fig. 3*B, left*), confirming the specificity of the effect. There was a strict correlation between the action exerted by Pin1 on the steady-state levels of RARα and the functional activity of the peptidyl-prolyl-isomerase. First, *Y23/A* was equally unable to decrease the levels of RARα (Fig. 3*B, right*), interact with the receptor (see Fig. 1*C*), and inhibit ATRA-dependent RARα transactivation (Fig. 2*C*). Second, the catalytically inactive *S67/E* protein did not alter the levels (Fig. 3*B, right*) or affect the transcriptional activity of RARα (Fig. 2*C*). Third, treatment of Pin1- and PML-RARα–, or RARα-transfected COS-7 cells with PiB partially reverted the down-modulation of the two retinoid receptors afforded by overexpression of the peptidyl-prolyl-isomerase (Fig. 3*C*). All these effects were independent of the presence of ATRA in the culture medium.

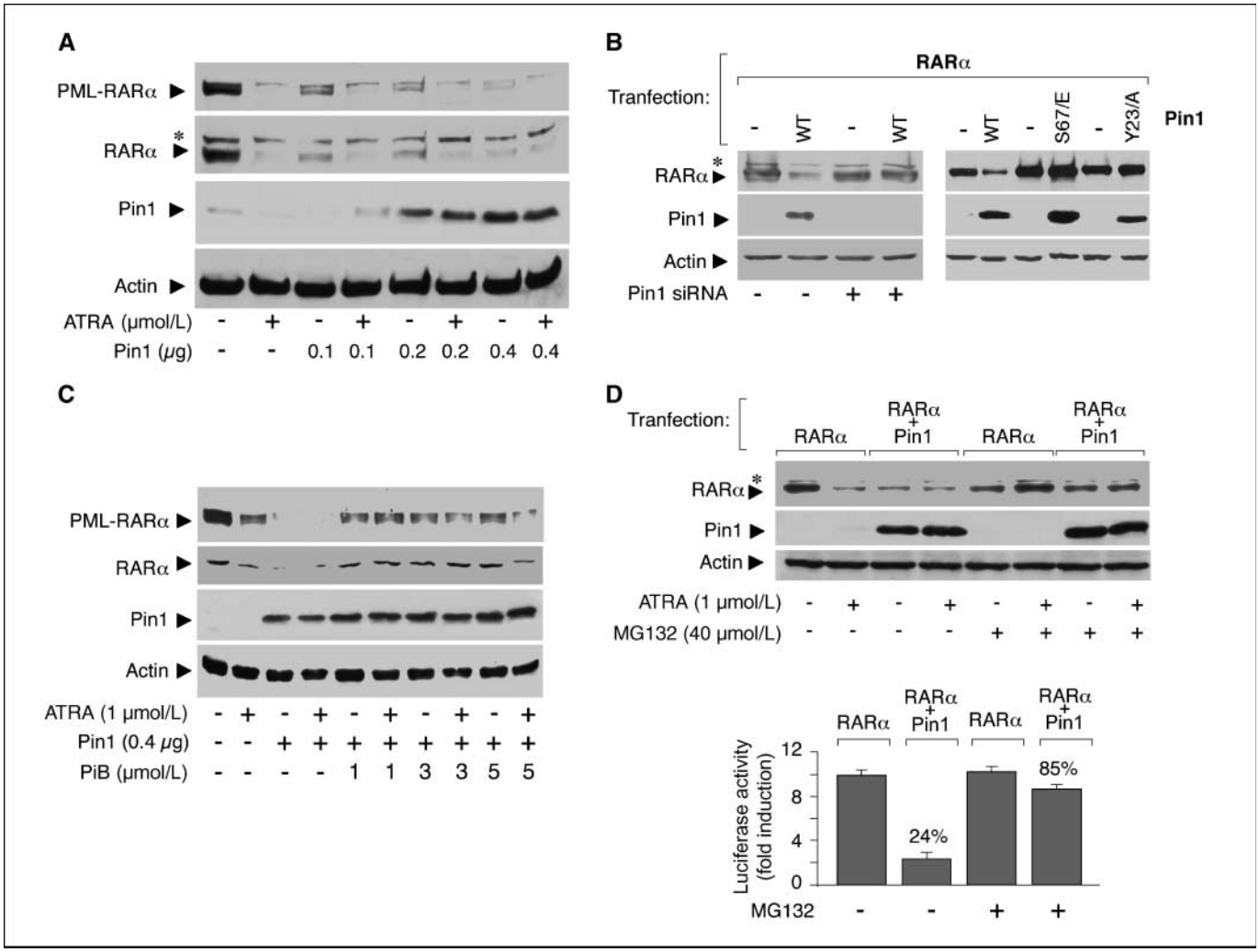

Figure 3. Pin1 overexpression decreases the steady-state levels of RARα and PML-RARα: effects of Pin1 silencing, pharmacologic inhibition, or functional inactivation and proteasome inhibition by MG132. *A,* COS-7 cells were transfected with RARα or PML-RARα (0.1 μg), the RARE-tk-Luc reporter (1 μg), and Pin1. Twenty-four hours after transfection, cells were treated with DMSO or ATRA (1 μmol/L) for a further 24 h. Western blots of RARα, Pin1, and actin are shown. *B, left,* COS-7 cells were transfected with RARα (0.1 μg) in the presence or absence of Pin1 (WT, 0.4 μg) and the anti-Pin1 siRNA (20 nmol/L). Twenty-four hours after transfection, Western blot analyses for RARα, Pin1, and actin were performed. *Right,* COS-7 cells were transfected with RARα (0.1 μg) in the presence of WT Pin1 or Pin1 mutants (S67/E, Y23/A, 0.4 μg). Western blot analyses were conducted as in *A. C,* COS-7 cells transfected as in *B*. Twenty-four hours after transfection, cells were treated with vehicle or ATRA in the presence or absence of the indicated concentrations of PiB for another 24 h. *D, top,* COS-7 cells transfected as in *B*. Twenty-four hours after transfection, cells were treated with vehicle or ATRA in the presence or absence of MG132 (40 μmol/L) for another 8 h. Western blots of RARα, Pin1, and actin are shown. *Bottom,* COS-7 cells were transfected, treated as above, and luciferase activity was measured. Results are expressed in fold induction, which is the ratio of the luciferase activity measured in ATRA- and DMSO-treated cells. The values above the columns indicate the percentage of luciferase activity determined in RARα+Pin1 relative to RARα-transfected cells. *Columns,* mean of duplicate transfections; *bars,* SD. All the results are representative of at least two experiments. The asterisk on the left of *A, B*, and *D* indicates a nonspecific band recognized by the anti-RARα antibody.

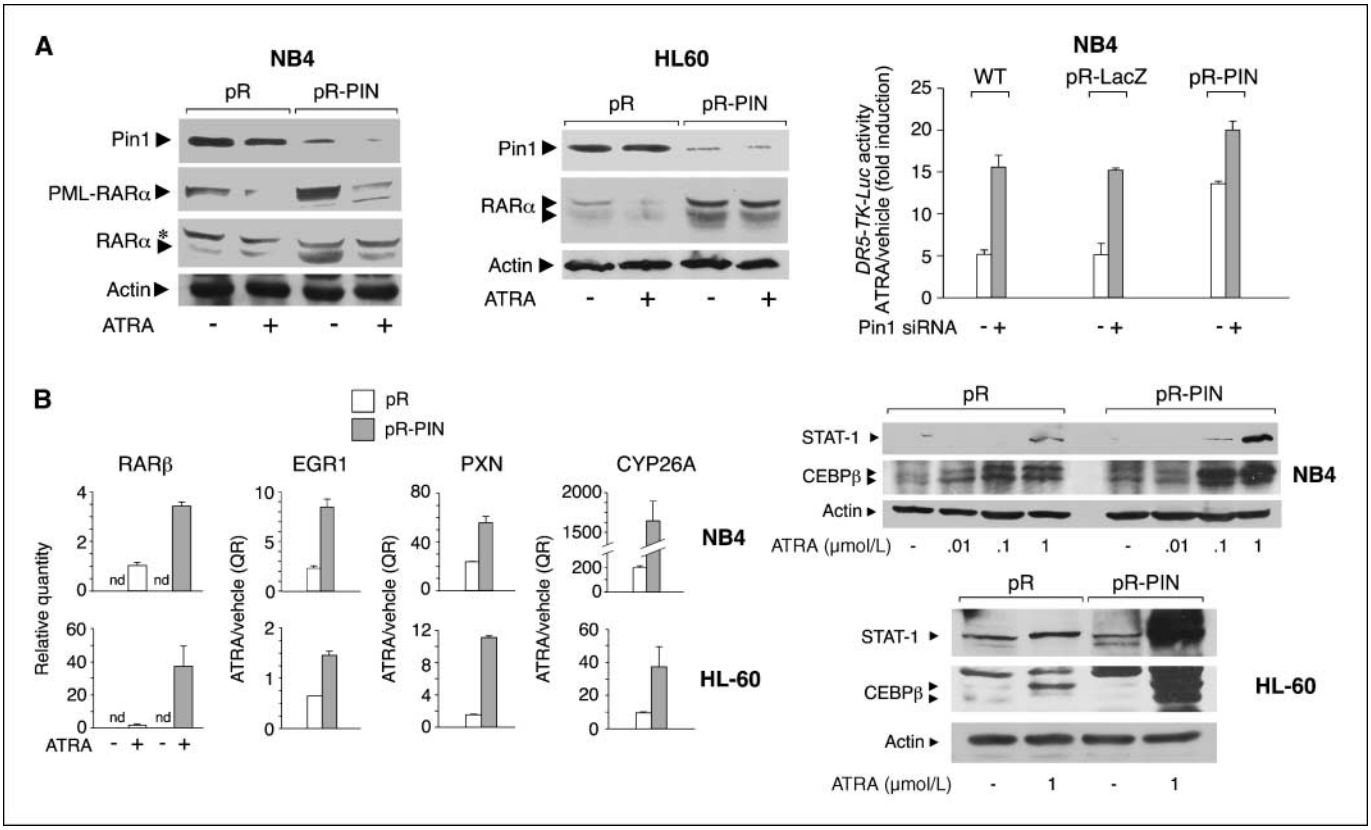

Figure 4. Effects of Pin1 silencing in NB4 and HL-60 cells. Plasmid expressing a shRNA targeting Pin1 (*pR-PIN-NB4* or *pR-PIN-HL-60*), an irrelevant shRNA (*pR-LacZ-NB4* or *pR-LacZ-HL-60*), or the corresponding empty vector (*pR-NB4* or *pR-HL-60*) were electroporated in NB4 and HL-60 cells. Stably transfected populations of each cell line were obtained. *A, left and middle,* cells were treated with DMSO or ATRA (0.1 μmol/L) for 24 h. Western blots of Pin1, RARα, and actin are shown. The asterisk on the left indicates a nonspecific band recognized by the anti-RARα antibody. *Right, WT, pR-Lacz-NB4*, or *pR-PIN-NB4* cells were electroporated with the *DR5-TK-Luc* reporter and renilla luciferase in the absence or presence of the anti-Pin1 siRNA (*siRNA A*, 20 nmol/L) used in Fig. 2*B*. Twelve hours after electroporation, cells were treated with DMSO or ATRA (0.1 μmol/L) for a further 24 h. Firefly luciferase activity was measured and normalized for renilla luciferase activity. Results are expressed in fold induction, which is the ratio of the normalized luciferase activity measured in cells treated with ATRA and DMSO. *Columns,* mean of duplicate transfections; *bars,* SD. *B, left graphs,* total RNA extracted from cells treated as in *A* was used for the amplification of the indicated transcripts by quantitative real-time RT-PCR. *Columns,* mean of two biological replicates consisting of independent cell cultures; *bars,* SD. *Right panels, pR-PIN-NB4, pR-NB4* or *pR-PIN-HL-60*, and *pR-HL-60* cells were treated with DMSO or the indicated concentrations of ATRA for 24 h. Western blots of STAT-1, cEBPβ, and actin are shown.

The decrease in RARα levels caused by Pin1 overexpression was due to proteasome-dependent degradation of the protein (Fig. 3*D*), because the proteasome inhibitor, MG132, reverted this effect. As expected (44), MG132 exerted a protective effect also in the case of ATRA-induced RARα degradation, regardless of Pin1 overexpression. Degradation of RARα is likely to be an important mechanism for the inhibitory effect exerted by Pin1 on ATRA-dependent transactivation of the receptor (Fig. 3*D, bar graph*). Indeed, treatment with MG132 of Pin1-transfected cells resulted in functional recovery of RARα. The same phenomena were observed if RARα was substituted for by PML-RARα (data not shown). Interestingly, Pin1 overexpression had no significant effect on the levels of the RAR partner protein RXR (Supplementary Fig. S2*B*).

Silencing of Pin1 causes stabilization of RARα/PML-RARα and sensitization of NB4 and HL-60 cells to ATRA. To establish the physiologic significance of Pin1 for the retinoid pathway, we silenced the corresponding gene in NB4 and HL-60 cells by electroporating a plasmid containing a shRNA targeting Pin1. Two stably transfected populations for each cell line were obtained: one expressing the specific shRNA (*pR-PIN-NB4* or *pR-PIN-HL-60*) and the other expressing the void vector (*pR-NB4* or *pR-HL-60*). *pR-NB4* and *pR-HL-60* expressed levels of Pin1 comparable with those observed in the corresponding parental cell lines (data not shown). Efficient silencing of Pin1 (>75% reduction in the levels of Pin1 protein) was observed in both control and ATRA-treated *pR-PIN-NB4* and *pR-PIN-HL-60* cells (Fig. 4*A*).

In basal conditions, suppression of Pin1 was accompanied by a significant elevation in the steady-state levels of RARα in both *pR-PIN-NB4* and *pR-PIN-HL-60* cells (Fig. 4*A*). Increased amounts of the protein in *pR-PIN-NB4* and *pR-PIN-HL-60* relative to *pR-NB4* and *pR-HL-60* cells were observed also after challenge with ATRA (0.1 μmol/L) for 24 hours. A similar effect occurred with the PML-RARα protein expressed in *pR-PIN-NB4* cells. No differences in the amounts of RARα and/or PML-RARα transcripts were observed in Pin1 silenced and control cell lines (data not shown). This is consistent with what was observed in COS-7 cells and suggests that suppression of Pin1 slows down constitutive degradation of RARα/PML-RARα. As expected, only residual binding of RARα to Pin1 was detected in pR-PIN-HL-60 cells, after immunoprecipitation with anti-Pin1 and/or anti-RARα antibodies (data not shown), supporting the specificity of the interaction between the two proteins.

Pin1 knockdown resulted in sensitization of NB4 and HL-60 cells to ATRA by a number of complementary experiments. As shown in Fig. 4*A*, relative to what is observed in the absence of the silencing

oligonucleotide, transfection of an anti-Pin1 siRNA in *WT* or *pR-LacZ-NB4* (stably transfected with an irrelevant shRNA targeting bacterial β-galactosidase) cells along with *DR5-TK-Luc* resulted in enhanced ligand-dependent transcription of the RARE-containing reporter. Moreover, comparison of shRNA-silenced *pR-PIN-NB4* with *WT* or *pR-LacZ-NB4* cells showed a significant increase of the ATRA-induced activation of the luciferase reporter. Finally, transfection of the siRNA targeting Pin1 in *pR-PIN-NB4* cells resulted in minor increases in ATRA-dependent luciferase activity, consistent with almost maximal silencing of Pin1 by stable expression of the specific shRNA. A similar trend of results was observed after conducting the same type of experiments in the HL-60 model (data not shown).

Our results were supported by analysis of the ligand-dependent transcription of selected retinoid-dependent genes (Fig. 4*B*). Four direct retinoid-target genes (*RARβ2, Egr1, PXN*, and *CYP26A*), containing functional RAREs were tested for their response to ATRA (0.1 μmol/L) in *pR-NB4/pR-PIN-NB4* or *pR-HL-60/pR-PIN-HL-60* cells, using quantitative real-time RT-PCR. Although quantitative differences in ATRA-dependent induction of *RARβ2, Egr1*, and *PXN* mRNAs were noticeable, *pR-PIN-NB4* and *pR-PIN-HL-60* responded to ATRA with a more robust increase in the amounts of the three transcripts than the corresponding control cells. CYP26 was much more inducible by ATRA in *pR-PIN-NB4* than in *pR-NB4* cells, whereas the effect was less significant in the *pR-PIN-HL-60/pR-HL-60* couple. In addition, we evaluated the effect of Pin1 silencing on cEBPβ and STAT-1, two proteins encoding transcription factors involved in the process of granulocytic maturation activated by ATRA (31). Even in this case, Pin1 down-regulation enhanced ATRA-dependent induction of the two proteins in both NB4 and HL-60 cells.

One of the functional consequences of cellular sensitization to retinoids by Pin1 silencing was enhanced myeloid maturation (Supplementary Fig. S3), as indicated by the differentiation markers NBT reductase, CD11b, CD11c, and CD38 (11). Augmented NBT reductase activity was observed in *pR-PIN-NB4* relative to *pR-NB4* cells treated for 4 days with increasing concentrations of ATRA. A similar effect occurred in the *pR-PIN-HL-60/pR-HL-60* couple, when the number of NBT reductase–positive cells was considered. Although a dose-dependent enhancement of NBT reductase was observed with concentrations of ATRA ranging from 0.1 to 10 μmol/L in *pR-PIN-NB4*, the effect was already maximal at 0.01 μmol/L ATRA in the *pR-PIN-HL-60* counterpart. Relative to the corresponding control, increases in the number of CD11b- and CD11c-positive *pR-PIN-NB4* cells were caused only by the lowest concentration of ATRA. Significant elevations of cell-associated CD11c [mean-associated fluorescence (MAF)] were evident in *pR-PIN-NB4* cells treated with 0.01 μmol/L ATRA or higher. In the *PIN-HL-60/pR-HL-60* couple, Pin1 silencing caused an increase in the number of CD11b-positive cells and in the amount of CD11b expression (MAF) only after treatment with 0.01 μmol/L ATRA. As to CD11c, the two parameters were elevated with all concentrations of ATRA. Although the vast majority of NB4 cells was CD38 positive, Pin1 silencing increased the amount of cell-associated CD38 (MAF) already at 0.01 μmol/L ATRA. A similar effect was not observed in the context of HL-60 cells.

Interestingly, a substantial proportion of *pR-PIN-HL-60* cells expressed both CD11b and CD11c in basal conditions, suggesting a more differentiated phenotype. A similar, albeit quantitatively lower, effect was observed with CD11c in *pR-PIN-NB4.* Because all the experiments described were conducted in complete medium, expression of myeloid markers in the absence of added ATRA can be explained by sensitization to endogenous serum retinoids (generally 0.001–0.005 μmol/L).

The effect of Pin1 silencing on the growth inhibitory action of ATRA is complex. In fact, silencing was associated with a significant decrease in the basal growth rate of NB4 (Supplementary Fig. S4*A*) and HL-60[5] cells, which complicated the interpretations of the data obtained after challenge with ATRA. Nevertheless, the data obtained in *pR-PIN-NB4* cells indicate that Pin1 down-modulation enhances the growth inhibitory response to ATRA after short exposure to suboptimal concentrations of the retinoid (Supplementary Fig. S4*B*). Notably, the antiproliferative effect exerted by ATRA in *pR-PIN-NB4* cells was not associated with evident signs of apoptosis up until 4 days of treatment.

Pharmacologic inhibition of Pin1 by PiB stabilizes *RARα* or *PML-RARα* and sensitizes myeloid leukemia cells to retinoids. To establish whether inhibition of Pin1 catalytic activity was equivalent to silencing of the corresponding gene, we performed combination studies in NB4 and HL-60 cells with PiB and ATRA. In NB4 cells, cotreatment with PiB and ATRA was more effective than ATRA alone in inducing the expression of the *EGR-1* and *PXN* genes (Fig. 5*A*), indicating sensitization. Sensitization was not limited to NB4 and extended to HL-60 cells (Fig. 5*B*) as well as to other targets of retinoid activity like cEBPβ and total or tyrosine-phosphorylated STAT-1 (ref. 31; Supplementary Fig. S5). PiB was just potentiating the differentiating activity of ATRA, as the compound, on its own, was devoid of any modulating action on the expression of the retinoid-dependent markers considered.

Increased responsiveness to ATRA translated into augmented granulocytic differentiation of both the NB4 and HL-60 blasts (Fig. 5*A* and *B*). Combined treatment with PiB and ATRA induced the appearance of a greater number of NBT reductase–positive NB4 and HL-60 cells relative to treatment with ATRA alone. A similar effect was observed in PR-9 cells after conditional expression of PML-RARα (Supplementary Fig. S6*C*). The combination of PiB+ATRA was also more efficient than the single components in increasing surface expression of CD11b and CD11c in both NB4 and HL-60 cells (Fig. 5*A* and *B*).

Treatment of NB4 cells with PiB potentiated not only the differentiating but also the antiproliferative action exerted by ATRA (Fig. 5*A*). Indeed, whereas PiB alone had no significant effect on the growth of this cell line, treatment with the Pin1 inhibitor and ATRA (0.01 and 1 μmol/L) enhanced the antiproliferative effect of the retinoid. Noticeably, enhancement was evident after 3 and 4 days of continuous cotreatment even at the lowest concentration of ATRA, which, on its own, had only a marginal antiproliferative effect. Increased growth inhibition by the combination of PiB and ATRA was associated with an increase in the proportion of cells in the G_1 phase and a parallel decrease in the proportion of cells transiting through the S phase of the cycle (Supplementary Fig. S7). A slightly different situation was observed in the HL-60 model (Fig. 5*B*). Monotreatment of HL-60 cells with PiB or ATRA for 4 days resulted in a similar and sustained growth-inhibitory action. Once again, combinations between PiB and ATRA enhanced the growth-inhibitory effects observed with either compound. Interestingly, a synergistic interaction between PiB and ATRA in terms of growth inhibition was observed also in PR9 cells expressing PML-RARα (Supplementary Fig. S6*D*).

[5] Maurizio Gianni, unpublished observations.

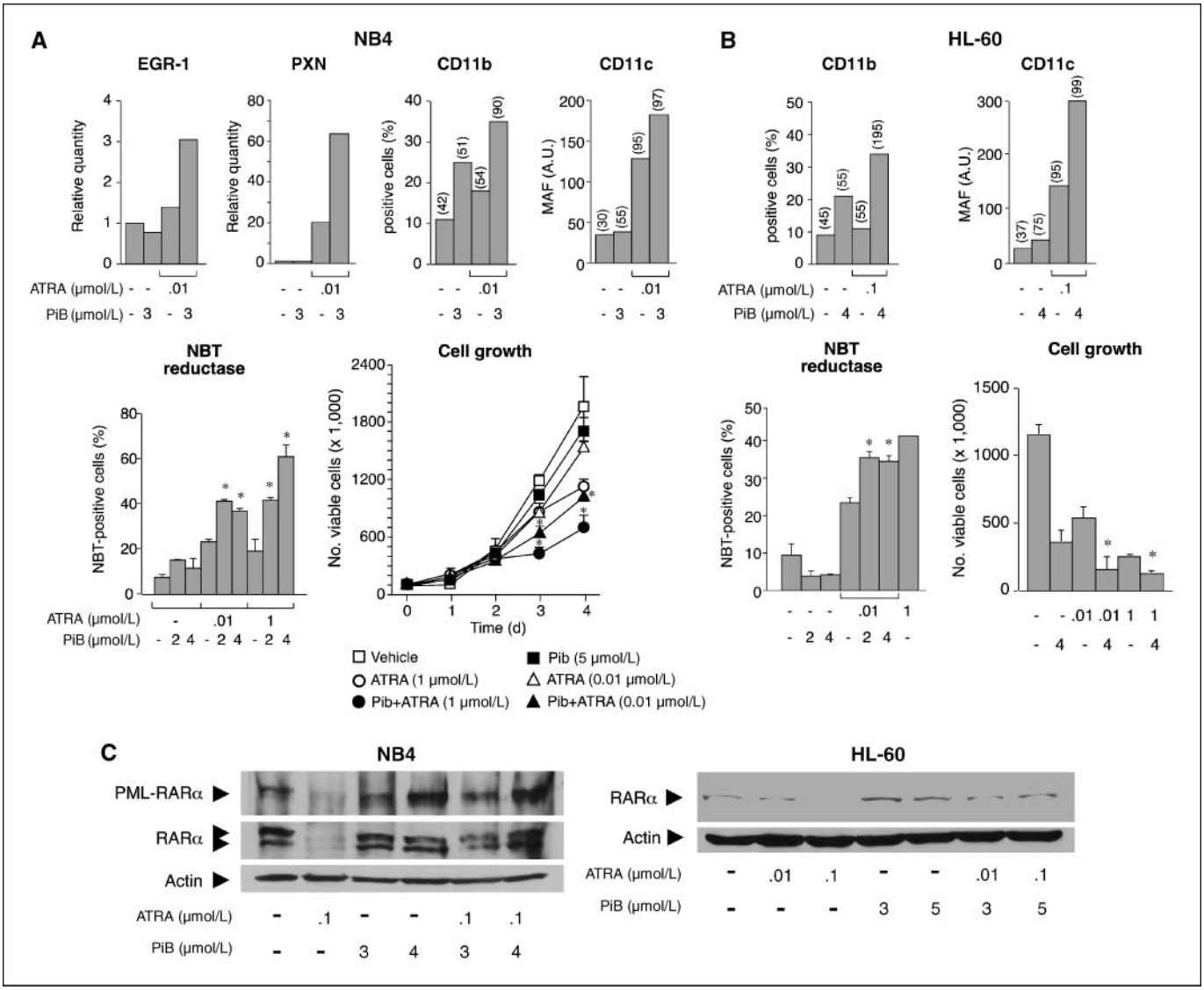

Figure 5. Effects of Pin1 inhibition by PiB in NB4 and HL-60 cells. *A* and *B,* NB4 and HL-60 cells (150,000/mL) were treated with the indicated concentrations of PiB and/or ATRA for 4 d, unless otherwise stated. The amounts of EGR-1 and PXN transcripts (*A, top left*) were measured by real-time RT-PCR as in Fig. 4*B*. *Columns,* mean of two independent cell cultures; *bars,* SD. The number of NBT reductase–positive cells was also determined (*A* and *B, bottom left*). An aliquot of the same cell populations was also subjected to FACS analysis for the indicated surface markers. The percentage of CD11b-positive cells and the amount of cell-associated CD11c (*MAF*) are shown by the bar graphs (*A* and *B*). Conversely, the numbers in parenthesis represent the MAF (CD11b) and percentage of positive cells (CD11c). The number of viable cells (*A* and *B, bottom right*) was counted manually after staining with trypan blue following treatment for 1 to 4 d in the case of NB4 cells and only 4 d in the case of HL-60 cells. In these experimental conditions, the viability of cells is ≥90% in all cases. *C, right and left,* NB4 and HL-60 cells were treated for 24 h as indicated. Western blots of RARα and β-actin are shown. All the results are representative of at least two experiments.

Sensitization to retinoids by PiB required the presence of a functionally active retinoid signaling pathway (Supplementary Fig. S8*A* and *B*). Indeed, the two retinoid-resistant cell lines, HL-60R, expressing an inactive form of RARα, and NB4.007, showing constitutive degradation of PML-RARα (28, 45), were substantially refractory to PiB and/or ATRA, both in terms of NBT reductase activity and growth inhibition.

Relative to vehicle-treated cells, treatment of NB4 cells (Fig. 5*C*) for 24 hours with PiB caused significant increases in the basal levels of PML-RARα and a more limited effect on RARα. PiB protected cells from ATRA-induced degradation of the two receptors as well. Similar phenomena involving RARα were observed in HL-60 blasts treated with PiB alone or in combination with ATRA (Fig. 5*C*). Protection of ATRA-induced PML-RARα degradation by Pin1 inhibition was also verified in the PR9 cell line after expression of PML-RARα (Supplementary Fig. S6*A* and *B*).

PiB sensitizes freshly isolated AML blasts to ATRA. The effect of PiB was studied in blasts isolated from AML patients whose characteristics are summarized in Supplementary Table S1. For these studies, the same differentiation markers used for HL-60 and NB4 cells were considered.

The first patient was characterized by expression of Pin1, which was not modulated by ATRA alone or in combination with PiB (Fig. 6*A*). Treatment for 4 days with ATRA (0.1 μmol/L) increased the number of CD11b-positive cells and induced the amount of cell-associated CD11c or CD38 observed in control conditions. At higher concentrations (1 μmol/L), ATRA induced also STAT-1. Although devoid of any activity on its own, PiB (1 μmol/L)

potentiated the effects exerted by ATRA on CD11b, CD11c, and, to a lesser extent, on CD38. Although PiB enhanced retinoid-dependent induction of STAT-1, the effect was just additive, as up-regulation of the protein was already observed in cells treated with PiB alone. Interestingly, PiB protected AML blasts from ATRA-induced degradation of RARα. This supports the concept that at least part of the potentiating effect of Pin1 inhibition is explained by stabilization of the retinoid receptor.

The induction of the two retinoid-dependent transcription factors, cEBPβ and STAT1, was observed in the blasts of patient 2 after incubation for 4 days with the combination of PiB and ATRA (Supplementary Fig. S9). However, PiB, ATRA, and combinations thereof did not affect the levels of NBT reductase, CD11b, CD11c, or CD38 (data not shown). PiB and ATRA alone or in combination had no effect on Pin1 expression. In the APL patient 3, whose blasts express similar levels of Pin1 as patient 1 (Supplementary Fig. S9), PiB enhanced ATRA-dependent induction of NBT reductase activity (Fig. 6*B*). In both vehicle- and ATRA-treated cells, Pin1 treatment was associated with an increase in the steady-state levels of PML-RARα and RARα (Fig. 6*B*).

In patient 4, PiB potentiated all the retinoid-dependent responses considered (Fig. 6*C*). Indeed, the inhibitor enhanced the induction of NBT reductase, CD11b, and CD11c afforded by treatment for 5 days with two concentrations of ATRA (0.1 and 1 μmol/L). A similar effect was observed after 1 and 5 days of treatment in the case of cEBPβ and STAT-1. All these PiB-dependent effects were accompanied by protection from ATRA-induced degradation of RARα. In the case of patient 5, combinations of PiB (1 μmol/L) and ATRA (1 μmol/L) were more effective than the single compounds in increasing the number of CD11c-positive cells and in inducing the expression of cEBPβ or STAT-1 (Supplementary Fig. S9). Interestingly, patient 6, representing an evolution of a CMML to AML, was associated with complete refractoriness to ATRA and consequently to PiB as well (data not shown).

Discussion

The results of this report indicate that Pin1 is a component of the transcriptional complex containing RARα, the major retinoid receptor expressed in the myeloid lineage. Pin1 binds to the

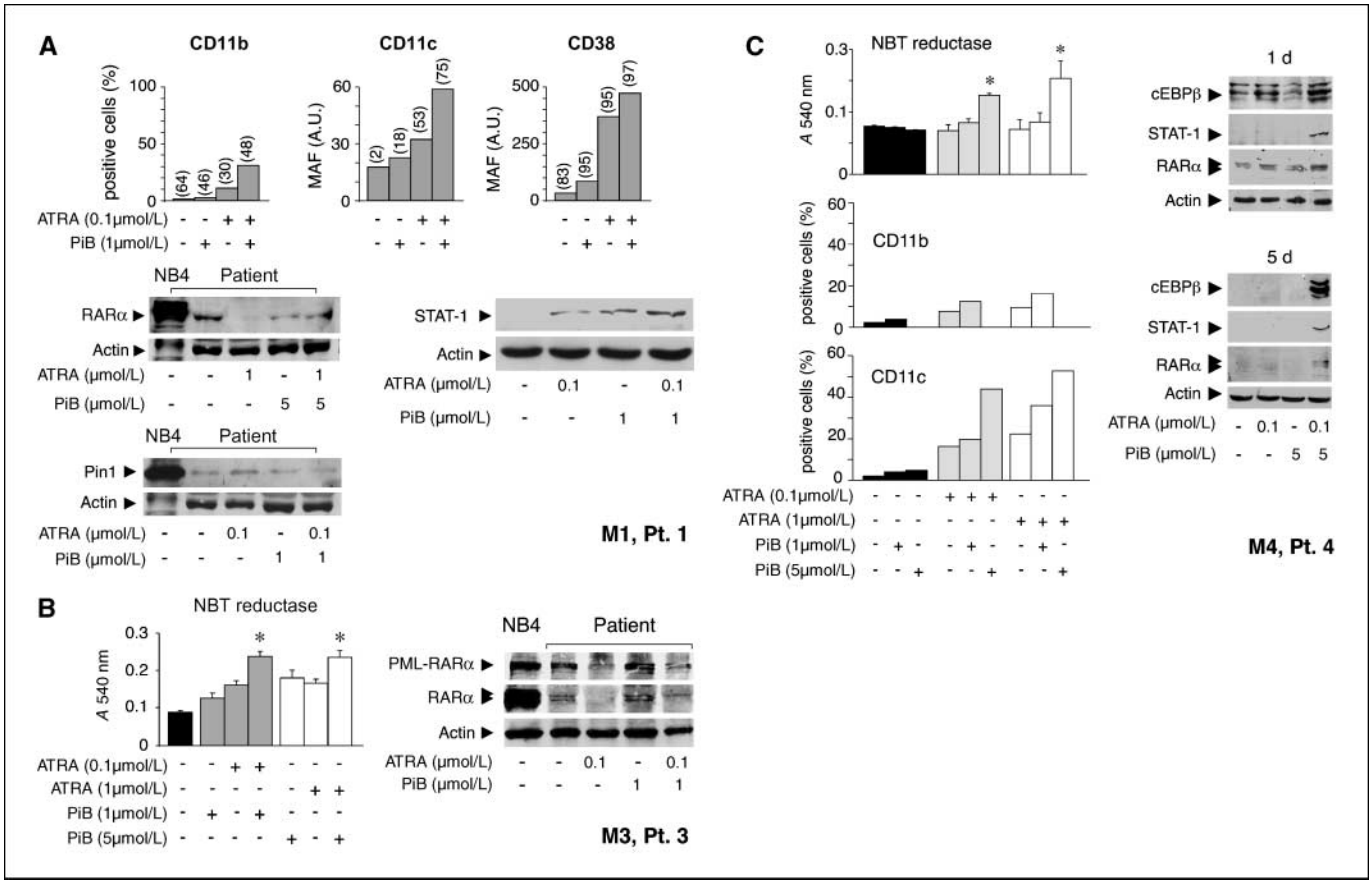

Figure 6. Effects of Pin1 inhibition by PiB in freshly isolated AML cells. *A,* freshly isolated blasts from patient 1 (500,000 cells/mL) were treated with ATRA and/or PiB for 4 d. The percentage of CD11b-positive cells and the amount of cell-associated CD11c and CD38 surface marker (*MAF*) was determined by FACS analysis (*bar graphs, top*). The CD11b MAF and the percentage of CD11c- and CD38-positive cells are indicated by the numbers in parenthesis. Western blots of STAT-1, RARα, Pin1, and β-actin are shown. *B,* blasts from patient 3 were treated with the indicated concentrations of ATRA and/or PiB for 4 d. The amount of NBT reductase activity is shown on the left. *Columns,* mean of three replicate cultures; *bars,* SD. *, significantly higher relative to the group treated with ATRA (Student's *t* test, $P < 0.01$). Western blots of RARα and β-actin are also shown. *C,* for the measurement of NBT reductase activity, blasts from patient 4 were treated as indicated for 7 d. *Columns,* mean of three replicate cultures; *bars,* SD. *, significantly higher relative to the group treated with ATRA (Student's *t* test, $P < 0.01$). The percentage of CD11b- and CD11c-positive cells was determined after 5 d of treatment by FACS analysis. The levels of STAT-1, RARα, CEBPβ, and β-actin proteins were determined by Western blot analysis after 1 and 5 d of treatment.

unliganded form of the receptor directly and binding is not influenced by ATRA. PML-RARα, the aberrant form of the receptor expressed in APL blasts, retains the ability to interact with Pin1. Interaction with Pin1 requires phosphorylation of RARα, and most likely of PML-RARα too. Several phosphorylated Ser(Thr)-Pro motifs potentially involved in the binding to Pin1 are present throughout the sequence of RARα and PML-RARα. We are in the process of mapping the critical residues responsible for the interaction with the peptidyl-prolyl-isomerase, using phosphorylation mutants and deletions of RARα and PML-RARα. Interestingly, it was recently shown that PML (46) is a client protein of Pin1, hence it is likely that not only the RARα but also the PML moiety of PML-RARα is important for the binding to the peptidyl-prolyl-isomerase. Because the interaction between RARα/PML-RARα and Pin1 is ligand independent, crucial phosphorylation sites must be targets of constitutive kinases acting on the unliganded form of the receptors (39). As to the other partner in this interaction, the WW domain of Pin1, which lies upstream of the peptidyl-prolyl-isomerase catalytic region, is necessary and sufficient for the binding to RARα and PML-RARα. Within this domain, the Y/23 residue is critical for the interaction with either retinoid receptor (33).

Pin1 is a negative regulator of RARα functional activity. Our data are consistent with the idea that the interaction with Pin1 is instrumental in routing unliganded RARα and/or PML-RARα along the proteasome-dependent degradation pathway. We propose that Pin1 lies upstream of the ubiquitination machinery,[5] inducing conformational changes to RARα that make the receptor accessible to the ubiqutin ligase/transferase complex and subsequent proteosome-dependent degradation. Although Pin1 is not directly involved in the ligand-dependent degradation of RARα or PML-RARα, the peptidyl-prolyl isomerase affects the process indirectly. Indeed, silencing or inhibition of Pin1 causes an increase in the steady-state levels of both receptors not only in basal conditions but also after treatment of NB4 and HL-60 cells with ATRA. Although we cannot rule out modification of other Pin1-dependent cellular effects by silencing/inhibition of the peptidyl-prolyl isomerase, stabilization of RARα and/or PML-RARα may be at the basis of the differences in the response to retinoids observed in the AML-derived HL-60 or NB4 cell lines and in freshly isolated AML blasts. Indeed, constitutive silencing of Pin1 causes enhanced expression of various retinoid-regulated genes, as well as a more rapid and sustained differentiating response to the retinoid. We propose that this is the direct consequence of a net increase of the RARα and/or PML-RARα pool(s), which is susceptible to be activated by ATRA.

RARα stabilization by Pin1 silencing/inhibition and consequent increases in the steady-state levels of the protein may entirely explain the observed potentiation of ATRA-dependent differentiation and/or growth inhibition in AML cellular contexts lacking PML-RARα. Conversely, the observation that enhanced differentiation of the APL blast is associated with stabilization not only of RARα but also of PML-RARα challenges the mainstream views on the molecular mechanisms underlying the response of the leukemic cell to ATRA, as the aberrant receptor is generally considered to be a suppressor of the retinoid-dependent network of genes. However, a number of considerations are relevant in this context. The results obtained after Pin1 silencing/inhibition are not the first instance in which stabilization of PML-RARα is associated with increased sensitivity of APL cells to retinoid-induced differentiation. In fact, we reported the same type of observation also after cotreatment of NB4 cells with STI571/Gleevec (6). In reference to this, there are a number of indirect pieces of evidence indicating that the responsiveness of APL blasts to the cytodifferentiating activity of ATRA and/or other retinoids may involve transcriptional activation of PML-RARα. First, PML-RARα is an efficient ligand-dependent transcriptional activator of several retinoid-dependent genes, in addition to exerting suppressive effects on RARα activity. Second, PLZF-RARα, another fusion protein present in a minority of ATRA-refractory APL cases, retains a suppressive action on RARα, but is largely devoid of ligand-dependent transcriptional activity on RARE-containing target genes. Third, overexpression of PML-RARα, but not PLZF-RARα, induces retinoid responsiveness in the U937 AML cell line (47). Finally, it must be considered that mutations in the ligand-binding site of PML-RARα knock down the transactivating properties of the receptor and are associated with resistance to the retinoid in relapsed APL patients (48). Taken together, these data suggest that the differentiating effect of ATRA on APL cells may not simply be explained by relief of the suppressive action of PML-RARα on the retinoid-dependent pathway. Hence, the beneficial effect of ATRA may also involve an active participation of PML-RARα, functioning as a ligand-dependent transcriptional activator of retinoid-dependent genes important for the granulocytic maturation of the APL blast. Clearly, we are not purporting the idea that stabilization of PML-RARα is the only mechanism at work in our APL-derived experimental paradigms. It is possible that Pin1 silencing or inhibition causes structural alterations of PML-RARα affecting not only its proteasome-dependent degradation but also its ligand-dependent transactivaton. These structural changes may enhance the transcriptional activity of the aberrant receptor, perhaps by destabilization of the interactions with corepressor proteins, and result in the potentiation/sensitization effects observed in NB4 and freshly isolated APL blasts.

Although differences in the modulation of single response markers are observed, general sensitization of NB4 and HL-60 cells to the differentiating action of ATRA is similar after silencing and pharmacologic inhibition of Pin1 with PiB. Indeed, combined treatment of NB4 and HL-60 cells with PiB and ATRA is associated with enhanced expression of the same retinoid-regulated myeloid transcription factors and markers targeted by Pin1 silencing. Pharmacologic combinations of PiB and ATRA are more effective than ATRA alone not only in the context of the retinoid-responsive NB4 and HL-60 cells but also in freshly isolated blasts of patients representing AML subtypes classified as M1 to M4. If this is expected in the case of the APL patient considered, the observation is very interesting and of potential therapeutic relevance in the other AML subtypes. In fact, our data indicate that Pin1 inhibition can boost the expression of a number of molecular determinants and markers of myeloid differentiation typically modulated by ATRA in AML cell types that are only partially responsive to the differentiating action of retinoids. It would be interesting to extend the number of AML patients analyzed to establish whether Pin1 targeting is more effective in potentiating the activity of ATRA in cases showing mutations of the nucleophosmin 1 (NPM1) protein, as the normal protein has been reported to act as a repressor of the retinoid signaling pathway (49).

In conclusion, beyond their significance at the basic level, the data contained in this report represent proof of principle that down-modulation of Pin1 activity is a viable strategy to increase the differentiating and antiproliferative activity of ATRA in APL and other types of AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/10/2008; revised 10/7/2008; accepted 10/29/2008.

Grant support: Enrico Garattini from the Associazione Italiana per la Ricerca contro il Cancro, Fondo Italiano per la Ricerca di Base Programma Internazionalizzazione, Fondazione "Italo Monzino," and Fondazione "Negri-Weizmann."

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Fiamma Mantovani, Gabriela Paroni, and Gianmaria Borleri for the reagents and technical help.

References

1. Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol 2006;46:451–80.
2. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008;111:2505–15.
3. Licht JD. Reconstructing a disease: What essential features of the retinoic acid receptor fusion oncoproteins generate acute promyelocytic leukemia? Cancer Cell 2006;9:73–4.
4. Melnick A, Licht JD. Deconstructing a disease: RARα, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood 1999;93:3167–215.
5. Grande A, Montanari M, Manfredini R, et al. A functionally active RARα nuclear receptor is expressed in retinoic acid non responsive early myeloblastic cell lines. Cell Death Differ 2001;8:70–82.
6. Gianni M, Kalac Y, Ponzanelli I, Rambaldi A, Terao M, Garattini E. Tyrosine kinase inhibitor STI571 potentiates the pharmacologic activity of retinoic acid in acute promyelocytic leukemia cells: effects on the degradation of RARα and PML-RARα. Blood 2001;97:3234–43.
7. Gianni M, Terao M, Norio P, Barbui T, Rambaldi A, Garattini E. All-trans retinoic acid and cyclic adenosine monophosphate cooperate in the expression of leukocyte alkaline phosphatase in acute promyelocytic leukemia cells. Blood 1995;85:3619–35.
8. Gianni M, Terao M, Zanotta S, Barbui T, Rambaldi A, Garattini E. Retinoic acid and granulocyte colony-stimulating factor synergistically induce leukocyte alkaline phosphatase in acute promyelocytic leukemia cells. Blood 1994;83:1909–21.
9. Pisano C, Kollar P, Gianni M, et al. Bis-indols: a novel class of molecules enhancing the cytodifferentiating properties of retinoids in myeloid leukemia cells. Blood 2002;100:3719–30.
10. Kamashev D, Vitoux D, De The H. PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. J Exp Med 2004;199:1163–74.
11. Parrella E, Gianni M, Cecconi V, et al. Phosphodiesterase IV inhibition by piclamilast potentiates the cytodifferentiating action of retinoids in myeloid leukemia cells. Cross-talk between the cAMP and the retinoic acid signaling pathways. J Biol Chem 2004;279:42026–40.
12. Bour G, Lalevee S, Rochette-Egly C. Protein kinases and the proteasome join in the combinatorial control of transcription by nuclear retinoic acid receptors. Trends Cell Biol 2007;17:302–9.
13. Gaillard E, Bruck N, Brelivet Y, et al. Phosphorylation by PKA potentiates retinoic acid receptor α activity by means of increasing interaction with and phosphorylation by cyclin H/cdk7. Proc Natl Acad Sci U S A 2006;103:9548–53.
14. Rochette-Egly C. Nuclear receptors: integration of multiple signalling pathways through phosphorylation. Cell Signal 2003;15:355–66.
15. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 2004;328:1–16.
16. Gianni M, Tarrade A, Nigro EA, Garattini E, Rochette-Egly C. The AF-1 and AF-2 domains of RAR γ 2 and RXR α cooperate for triggering the transactivation and the degradation of RAR γ 2/RXR α heterodimers. J Biol Chem 2003;278:34458–66.
17. Gianni M, Parrella E, Raska I, Jr., et al. P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RARα-mediated transcription. EMBO J 2006;25:739–51.
18. Garattini E, Gianni M, Terao M. Retinoids as differentiating agents in oncology: a network of interactions with intracellular pathways as the basis for rational therapeutic combinations. Curr Pharm Des 2007;13:1375–400.
19. Lippens G, Landrieu I, Smet C. Molecular mechanisms of the phospho-dependent prolyl *cis/trans* isomerase Pin1. FEBS J 2007;274:5211–22.
20. Lu KP, Finn G, Lee TH, Nicholson LK. Prolyl *cis*-trans isomerization as a molecular timer. Nat Chem Biol 2007;3:619–29.
21. De Nicola F, Bruno T, Iezzi S, et al. The prolyl isomerase Pin1 affects Che-1 stability in response to apoptotic DNA damage. J Biol Chem 2007;282:19685–91.
22. Mantovani F, Tocco F, Girardini J, et al. The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. Nat Struct Mol Biol 2007;14:912–20.
23. Lu KP, Suizu F, Zhou XZ, Finn G, Lam P, Wulf G. Targeting carcinogenesis: a role for the prolyl isomerase Pin1? Mol Carcinog 2006;45:397–402.
24. Zhu YY, Shi JM, Sun J, et al. [Expression of Pin1 in malignant hematopoietic cells and its relation with cell cycle]. Zhejiang Da Xue Xue Bao Yi Xue Ban 2004;33:500–3, 14.
25. Brondani V, Schefer Q, Hamy F, Klimkait T. The peptidyl-prolyl isomerase Pin1 regulates phospho-Ser77 retinoic acid receptor α stability. Biochem Biophys Res Commun 2005;328:6–13.
26. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 1991;77:1080–6.
27. Collins SJ. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood 1987;70:1233–44.
28. Fanelli M, Minucci S, Gelmetti V, Nervi C, Gambacorti-Passerini C, Pelicci PG. Constitutive degradation of PML/RARα through the proteasome pathway mediates retinoic acid resistance. Blood 1999;93:1477–81.
29. Robertson KA, Emami B, Collins SJ. Retinoic acid-resistant HL-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain that confers dominant negative activity. Blood 1992;80:1885–9.
30. Nervi C, Ferrara FF, Fanelli M, et al. Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARα fusion protein. Blood 1998;92:2244–51.
31. Gianni M, Terao M, Fortino I, et al. Stat1 is induced and activated by all-trans retinoic acid in acute promyelocytic leukemia cells. Blood 1997;89:1001–12.
32. Mantovani F, Piazza S, Gostissa M, et al. Pin1 links the activities of c-Abl and p300 in regulating p73 function. Mol Cell 2004;14:625–36.
33. Zacchi P, Gostissa M, Uchida T, et al. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature 2002;419:853–7.
34. Rochette-Egly C, Oulad-Abdelghani M, Staub A, et al. Phosphorylation of the retinoic acid receptor-α by protein kinase A. Mol Endocrinol 1995;9:860–71.
35. Wu Y, Li Q, Chen XZ. Detecting protein-protein interactions by Far western blotting. Nat Protoc 2007;2:3278–84.
36. Gaub MP, Rochette-Egly C, Lutz Y, et al. Immunodetection of multiple species of retinoic acid receptor α: evidence for phosphorylation. Exp Cell Res 1992;201:335–46.
37. Delva L, Bastie JN, Rochette-Egly C, et al. Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex. Mol Cell Biol 1999;19:7158–67.
38. Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ, Pangalos MN. The use of TaqMan RT-PCR assays for semiquantitative analysis of gene expression in CNS tissues and disease models. J Neurosci Methods 2000;98:9–20.
39. Rochette-Egly C, Adam S, Rossignol M, Egly JM, Chambon P. Stimulation of RAR α activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell 1997;90:97–107.
40. Shaw PE. Peptidyl-prolyl cis/trans isomerases and transcription: is there a twist in the tail? EMBO Rep 2007;8:40–5.
41. Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res 2002;43:1773–808.
42. Uchida T, Takamiya M, Takahashi M, et al. Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation. Chem Biol 2003;10:15–24.
43. Wolf G, Smas CM. Retinoic acid induces the degradation of the leukemogenic protein encoded by the promyelocytic leukemia gene fused to the retinoic acid receptor α gene. Nutr Rev 2000;58:211–4.
44. Zhu J, Gianni M, Kopf E, et al. Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor α (RARα) and oncogenic RARα fusion proteins. Proc Natl Acad Sci U S A 1999;96:14807–12.
45. Li YP, Said F, Gallagher RE. Retinoic acid-resistant HL-60 cells exclusively contain mutant retinoic acid receptor-α. Blood 1994;83:3298–302.
46. Reineke EL, Lam M, Liu Q, et al. Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. Mol Cell Biol 2008;28:997–1006.
47. Ruthardt M, Testa U, Nervi C, et al. Opposite effects of the acute promyelocytic leukemia PML-retinoic acid receptor α (RAR α) and PLZF-RAR α fusion proteins on retinoic acid signalling. Mol Cell Biol 1997;17:4859–69.
48. Gallagher RE. Retinoic acid resistance in acute promyelocytic leukemia. Leukemia 2002;16:1940–58.
49. Liu H, Tan BC, Tseng KH, et al. Nucleophosmin acts as a novel AP2α-binding transcriptional corepressor during cell differentiation. EMBO Rep 2007;8:394–400.