PRAM-1 Is a Novel Adaptor Protein Regulated by Retinoic Acid (RA) and Promyelocytic Leukemia (PML)-RA Receptor α in Acute Promyelocytic Leukemia Cells*

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The (t(15;17)) translocation, found in 95% of acute promyelocytic leukemia, encodes a promyelocytic leukemia (PML)-retinoic acid receptor α (RARα) fusion protein. Complete remission of acute promyelocytic leukemia can be obtained by treating patients with all-trans retinoic acid, and PML-RARα plays a major role in mediating retinoic acid effects in leukemia cells. A main model proposed for acute promyelocytic leukemia is that PML-RARα exerts its oncogenic effects by repressing the expression of retinoic acid-inducible genes critical to myeloid differentiation. By applying subtraction cloning to acute promyelocytic leukemia cells, we identified a retinoic acid-induced gene, PRAM-1 (PML-RARα target gene encoding an Adaptor Molecule-1), which encodes a novel adaptor protein sharing structural homologies with the SLAP-130/fyb adaptor. PRAM-1 is expressed and regulated during normal human myelopoiesis. In U937 myeloid precursor cells, PRAM-1 expression is inhibited by expression of PML-RARα in the absence of ligand and de novo superinduced by retinoic acid. PRAM-1 associates with other adaptors, SLP-76, and SKAP-55HOM, in myeloid cell lines and with protein tyrosine kinase lyn. By providing the first evidence that PML-RARα dysregulates expression of an adaptor protein, our data open new insights into signaling events that are disrupted during transformation by PML-RARα and induced by retinoic acid during de novo differentiation of acute promyelocytic leukemia cells.

In human acute promyelocytic leukemia (APL),¹ a specific translocation, t(15;17) (1), creates a promyelocytic leukemia (PML)-retinoic acid receptor α (RARα) fusion between the amino-terminal region of the PML locus (2–5) and the carboxyl terminus of the RARα (6, 7). The causal role of the PML-RARα fusion protein in APL has been demonstrated in transgenic mice (8), although its effects do not appear to confer a fully malignant phenotype in murine promyelocytes (9). Leukemic transformation is likely to initiate in a subpopulation of hematopoietic stem/progenitor cells (10). In these cells, PML-RARα confers resistance to growth factor starvation (10), presumably exerting its oncogenic effect through repression of the genetic program normally leading to full myeloid differentiation. Because external signals such as cytokines regulate limited expansion and maturation of hematopoietic progenitor cells in the bone marrow, PML-RARα may initiate leukemia transformation in these cells by disrupting differentiation signaling pathways. However, though cytosolic signalings in APL cell differentiation are getting increasing attention (11), genes whose expression is repressed by PML-RARα remain poorly identified (9). Complete remission of APL can be obtained by treating patients with all-trans retinoic acid (ATRA) (12–15), which induces full differentiation of leukemic cells (15). This suggests that pharmacological levels of ligand can reverse the oncogenic effects of PML-RARα. It is likely that PML-RARα mediates the sensitivity of APL blasts to ATRA-induced differentiation. Indeed, ligand binding to the fusion protein induces the release of transcriptional repression (16), and PML-RARα may have an ATRA-dependent activator function (17). However, little is known about specific signaling through which ATRA induces differentiation.

Antigens binding to T and B cell receptors initiate intracellular signaling events leading to clonal expansion of reactive cells. Unlike growth factor receptors, T cell receptor and B cell receptor lack intrinsic tyrosine kinase activities. Instead, antigen receptor engagement results in activation of cytoplasmic protein tyrosine kinases (PTK). Among the substrates of the antigen receptor PTKs are members of a class of proteins that mediate protein-protein interactions, known as adaptors (18,

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The abbreviations used are: APL, acute promyelocytic leukemia; PML, promyelocytic leukemia; RA, retinoic acid; RARα, RA receptor α; ATRA, all-trans retinoic acid; PTK, protein tyrosine kinase; SH, Src homology domain; bp, base pair(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.
19. With multiple binding sites and the potential to create combinations of multiprotein complexes, adaptors are well suited to integrate signals from surface receptors. Little is known about adaptor proteins in myeloid cells (18).

Like APL patient blasts, NB4 leukemic cells carry the t(15; 17) and undergo ATRA-induced granulocytic differentiation (20). By applying a subtraction cloning approach to these cells, we identified a novel ATRA-inducible gene designated PRAM-1 (PML-RARα target gene encoding an Adaptor Molecule)-1. The predicted PRAM-1 protein contains proline-rich and SH3-like domains and shares structural homologies with the adaptor SLAP-130/fyb (21, 22). Ectopic expression of PML-RARα in U937 myeloid precursor cells inhibits PRAM-1 expression, which is superinduced upon treatment with ATRA. Furthermore, PRAM-1 is expressed and regulated early during in vitro differentiation of primary human hematopoietic stem/ progenitor cells. Finally, PRAM-1 interacts with known partner molecules of SLAP-130/fyb, such as SLP-76 and SKAP-HOM, as well as with the PTK lyn. These observations point to PRAM-1 as an adaptor protein with potentially important roles in leukemogenesis and myeloid differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture, and Differentiation—**NB4 (20), NB4.306 (23), and U937-PB9 and U937-MT cells (17) were cultured in RPMI 1640 medium with 10% fetal bovine serum (Life Technologies, Inc.) and 2 mm L-glutamine (Life Technologies, Inc.). COS-7 cells were grown in 9-cm Petri dishes in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. Cell viability was estimated using standard trypan blue dye exclusion. Cells were grown at 37 °C in a humidified atmosphere of 5% CO2.

Exponentially growing NB4, NB4.306, and U937-derived cells (1 × 106 cells/ml) were seeded at 2 × 105 cells/ml 16 h prior to ATRA treatment. U937-PB9 and U937-MT cells were used for induction of PML-RARα expression or as control, respectively. Cells were cultured either with or without varying concentrations of ZnSO4 (Sigma) and ATRA (Sigma). Differentiation was assessed by (i) the percentage of cells with nitro blue tetrazolium (Sigma) deposits, as described (24), and (ii) cell morphology under light microscopy on cytosin slides stained with May-Grünwald-Giemsa (Sigma).

**Human Cell Preparation and Liquid Culture—**Cord blood cells were obtained with informed consent from healthy donors at the end of full term deliveries at the Saint-Vincent de Paul Hospital (Paris, France). Sample preparation (achieving a purity of ≥ 1% CD34+ ) and CD34+ cell culture were conducted as described (25). Primary APL cells expressing the PML-RARα gene were obtained from patients diagnosed at Saint-Louis Hospital (Paris, France) and kindly provided by C. Chomienne. Cells were purified and cultivated with 10−7 M ATRA as described (14). 5 days after treatment, cells were tested for differentiation (>80% nitro blue tetrazolium-positive). Mononuclear cells and granulocytes were isolated from peripheral blood using a Ficoll Paque (Amersham Pharmacia Biotech) density gradient. Monocytes were further purified from lymphocytes using an indirect labeling system followed by a magnetic separation on a mac column (Myltenyi Biotec). Cell purity was assessed by May-Grünwald-Giemsa stain. Cell purity was assessed by May-Grünwald-Giemsa stain.

**PRAM-1 cDNA Cloning—**A cDNA fragment isolated from an ATRA-treated NB4 cDNA libraryb contained 1063 bp corresponding to the 3′ end of PRAM-1 (17). This fragment was used to search expressed sequence tag data bases (GenBankTM/EMBL and Incyte Genomics Lifeseq®) and, in combination with RACE-PCR reactions using HL-60 RACE-ready cDNA (CLONTECH), allowed us to generate 1800 bp of continuous open reading frame sequence. A 494-bp 5′ cDNA sequence was amplified by RACE-PCR from ATRA-treated NB4 RNA (with 5 × 10−4 M for 48 h) using the Marathon cDNA amplification kit (CLON-TECH) with a 5′-CCCTGGAACCCGATGCGGAGCAGCCG-3′ primer. Sequence homologies were identified using the Fasta and BLAST programs by searching GenBankTM and EMBL, and the Incyte Genomics Lifeseq® data base. Sequence information from many different Incyte Genomics data base clones was used to confirm and extend the results of RACE-PCR experiments. For example, clone 3419079H1 is 100% identical to the 3′ terminal end of the final PRAM-1 RACE product clone. Using the Marathon cDNA and the Advantage-GC polymerase (CLONTECH), a contiguous cDNA was generated by reverse transcription-PCR amplification of PRAM-1 from ATRA-treated NB4 RNA using 5′-GGCCCGACCTGCGGTCCACCTACTC-3′ and 5′-TGGCTGT- CTTGTGGCCCCAAGCTACC-3′ primers. PCR products were subcloned into pT-Adv (CLONTECH) and sequenced. All PCR-generated cDNAs were confirmed by multiple, independent derivations from RNA templates.

**Sequence and Computer Analysis—**Plasmid DNA was purified through Nucleobond columns (Macherey-Nagel), and double-stranded DNA templates were sequenced using PhleuScript and internal primers. Sequence analysis and alignments were obtained using the GCG program (Genetics Computer Group, Inc.). Sequence homologies were identified using the FASTA and BLAST programs by searching data banks as above and, in the case of translated sequences, by searching Swiss-Prot, PIR/NBRF, and SPTREMBL data banks.

**Plasmid Constructs—**The PRAM-1 coding sequence was subcloned into a pc8β Plus-derived expression vector (26) under the control of the zinc-inducible sheep metallothionein promoter from the pMT-10 vector (27), resulting in the pMT-PRAM-1 vector. The PRAM-1 coding sequence was also inserted into the pcT4 vector (28), to direct the expression of PRAM-1 tagged with the F domain of the human estrogen receptor at its amino terminus. SLP-76 and SKAP-HOM cDNAs were inserted into the pE-BOS expression vector (29, 30), at a site located 3′ to the sequences encoding the F-plant.

**Northern Blots—**Total RNA extraction and hybridization were as described (25). Human RNA Master Blot and human immune system multiple tissue Northern blot II were from CLONTECH. The PRAM-1 probe corresponded to the 3′ end (1063 bp) of the PRAM-1 cDNA. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 574-bp PCR product obtained using 5′-ATACACATCTTCCAGGAG-3′ sense and 5′-CCTGGTCACCCCTTCTG-3′ antisense primers. Autoradiography was quantified and counted using a Storm 860 phosphorimaging system (Amersham Pharmacia Biotech).

**Semi-quantitative Reverse Transcription-PCR—**Total RNA from CD34+ cells induced to differentiate toward the myeloid lineage was extracted at different times using a RNeasy mini kit (Qiagen). Reverse transcription was performed using the Advantage-2 cDNA synthesis kit (CLON-TECH). Expression of the PRAM-1 and control β-actin mRNA in differentiating cells was analyzed by semi-quantitative PCR using a Gene Amp PCR System 9600 (PerkinElmer Life Sciences) with the following primers corresponding to distinct exon sequences: sense (5′-CCTCAG-TTCAGAGCCAGCGAGG-3′) and antisense (5′-CCACAGGGGATGTGGTGTTTCCAGG-3′) for PRAM-1; sense (5′-CCTGGCCCTTGGCC-3′) and antisense (5′-GGCTTGGTACCGTACGCT-3′) for β-actin. PCR cycles were conducted with (i) denaturation for 45 s at 94 °C, (ii) annealing for 45 s at 60 °C, and (iii) extension for 1 min at 72 °C, followed by an extension final step at 72 °C for 7 min. The number of PCR cycles was determined for PRAM-1 (35 cycles) and β-actin (25 cycles) to ensure quantification of the PCR products in a linear range of amplification. The β-actin PCR reaction was performed for each sample prepared at each day of the kinetic to determine the dilutions needed to provide equal amounts of reverse transcription products for all samples. Aliquots of each PCR reaction were electrophoresed, transferred onto nylon filters (Hybond-N, Amersham Pharma- cia Biotech), and hybridized with the PRAM-1 (5′-CGCGCAAGCCA- TCTCCTCAATGTG-3′) and β-actin (5′-ACCCCGTGCTGCTGACCGAGG- 3′) P32-labeled probes as described (29). Autoradiography was quantified using a Storm 860 phosphorimaging system (Amersham Pharmacia Biotech).

**Antibodies—**An amino-terminal peptide (ESHDQDFRSIIAKFKQ) was synthesized according to the deduced amino acid sequence of the PRAM-1 protein and coupled to keyhole limpet hemocyanin through a cysteine residue added to the carboxyl-terminal amino acid of the peptide (Eurogentec). Rabbit sera (1PNP) were collected before (pre-immune) and 3 months after the initial injection and three booster immunizations (Eurogentec). A polyclonal sheep antiserum (Elmira Biologicals) was raised against the carboxyl-terminal 134 amino acids of human PRAM-1 fused with glutathione S-transferase (generated by cloning the corresponding PRAM-1 coding sequence into the pGEX vector (Amersham Pharmacia Biotech)). The monoclonal antibody directed against the F domain of human estrogen receptor was as described (28). Anti-Tyr(P) monoclonal antibody 4G10 was purchased from Upstate Biotechnologies, Inc., anti-Flag monoclonal antibody M2 was purchased from International Biotechnologies, Inc., and anti-lyn was purchased from Santa Cruz Biotechnology, Inc. Anti-SLP-76 sheep antiserum 0083 and anti-SKAP-HOM rabbit antiserum were as described (29, 30).
Fig. 1. PRAM-1 is induced in ATRA-treated NB4 cells. A, time course in response to 5 × 10⁻⁸ M ATRA in NB4 cells. Where indicated, de novo protein synthesis was inhibited by pretreating cells for 30 min with 10 μg/ml cycloheximide (CHX) followed by an additional 8 h in the absence (O) or presence (S) of ATRA. B, PRAM-1 expression correlates with the capacity of NB4 cells to differentiate. NB4 and NB4.306 cells cultured with 10⁻⁶ M ATRA were harvested after 0, 24, and 48 h. The percent of viable (a) and nitro blue tetrazolium-positive (b) cells is shown, as well as the level of PRAM-1 mRNA expression (c). Northern blots were performed using 5 μg (A) or 3 μg (B) of total RNA. Top and bottom panels show signal obtained with PRAM-1 and GAPDH (to control for RNA quantities) probes. C, cDNA and deduced amino acid sequences of the human PRAM-1. Nucleotide residues are numbered in the 5' to 3' orientation, and amino acids in the reading frame are designated by one-letter codes. Kozak underlines indicate the stop codon. The eight proline-rich repeats are underlined, and the asterisk indicates the stop codon. The eight proline-rich repeats are underlined in bold. Proline residues within motifs that bind SH2 domains are shaded.

In Vivo Expression and Protein Extracts—COS-7 cells were transfected using calcium phosphate coprecipitation (31) of 0.5-μg DNA vectors (adjusted to 14 μg per 9-cm Petri dish with pBluescript carrier DNA). The medium was changed after 16 h, and for zinc-induced expression of PRAM-1, 75 μM ZnSO₄ was added to the medium. Thirty-six hours after transfection, COS-7 cells were harvested, washed once in phosphate-buffered saline, and resuspended in lysis buffer (0.4 M NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.9, 20% glycerol, 5 mM diethiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and a protease inhibitor mixture (Sigma)). ATRA-treated NB4 cells were washed once in phosphate-buffered saline and resuspended in lysis buffer after two freeze-thaw cycles in liquid nitrogen, the resulting cell lysates were cleared by centrifugation at 4 °C.

Immunoprecipitation—One-step preclearing (30 min, 4 °C) with GammaBind G-Sepharose (Amersham Pharmacia Biotech), antibodies (1 μl of antigen serum or 1 μl of ascites fluid) were added to the cell protein extract, in a binding buffer adjusted to 20 mM Tris-HCl, pH 7.5, 0.25 mM NaCl, and 0.1% Nonidet P-40. After 90 min at 4 °C, 15 μl of GammaBind G-Sepharose suspension were added for an additional hour. Sepharose beads were then washed three times in 0.5 ml of the binding buffer. After 5 min of boiling in Laemmli buffer, samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Background immunoreactivity was reduced by preincubating membranes with phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h. Western analysis was carried out with antibodies diluted 1:5,000 in phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20. Detection of antibody binding was achieved with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories). Enzymatic activity was detected using the chemiluminescence reagent plus kit (PerkinElmer Life Sciences) and autoradiography.
by Group I SH2 domains (35). PRAM-1 shares homologies with the hematopoietic-specific adaptor molecule SLAP-130/fyb (21, 22), with a 57% similarity concentrated in the SH3-like domains in the extreme carboxyl termini of the two proteins. Both PRAM-1 and SLAP-130/fyb contain a central, proline-rich region. Together, these data suggest that PRAM-1 is a novel adaptor protein.

**PRAM-1 mRNA Is Expressed and Regulated in Primary Hematopoietic Tissues**—PRAM-1 mRNA was mainly expressed in bone marrow and peripheral blood leukocytes (Fig. 2A), in freshly purified human granulocytes and monocytes, as well as, albeit to a much lesser extent, in lymphocytes (Fig. 2B). PRAM-1 mRNA was not detected in non-hematopoietic tissues, except in lung, which is known to contain a number of myeloid cells (data not shown). Purified CD34+ positive human cord blood cells expressed low levels of the PRAM-1 mRNA (Fig. 2C, b, lane 1), which increased gradually during myeloid differentiation, with maximal expression at the promyelocytic stage (Fig. 2C, b, lane 6). Analysis of leukemia cell lines assigned to specific hematopoietic lineages supported the notion that PRAM-1 is mainly expressed in myeloid cells (data not shown). Together, these results indicate that PRAM-1 mRNA is expressed and regulated during normal myelopoiesis.

**PML-RARα Inhibits, and ATRA Subsequently Superinduces, Expression of PRAM-1 mRNA in U937 Cells**—In contrast to NB4 cells, PRAM-1 mRNA was not induced in NB4.306 cells, suggesting that an intact PML-RARα is required for ATRA to regulate PRAM-1 mRNA expression. To explore further the role of PML-RARα in the expression of PRAM-1 mRNA, we used cell lines generated by stable transfection into human U937 myeloid precursor cells of an zinc-inducible vector without an exogenous cDNA (U937-MT) or driving the PML-RARα cDNA (U937-PR9) (17). Zinc-induced expression of the PML-RARα protein in U937-PR9 cells resulted in an 8-fold decrease of PRAM-1 mRNA expression (Fig. 3A, lane 2 versus lane 1). Subsequent ATRA treatment of these cells resulted in a 60-fold increase of PRAM-1 mRNA expression at 24 h (Fig. 3A, lane 6 versus lane 2). No repression was observed in zinc-treated U937-MT cells (Fig. 3A, lane 8 versus lane 7), and a 3-fold increase was observed in these cells with ATRA treatment (Fig. 3A, lane 12 versus lane 7), as expected, because U937 cells express an active retinoic acid receptor (36). U937-PR9 and U937-MT cells were then left untreated or treated with increasing concentrations of ZnSO4. Cells were either untreated or treated with 5 × 10^{-7} M ATRA for 12 h, followed by exposure to 5 × 10^{-7} M ATRA for different times. B, autoradiogram (left panels) and relative expression (right panel) of PRAM-1 mRNA in U937-PR9 and U937-MT cells treated with increasing concentrations of ZnSO4. Cells were either untreated or treated with 5 × 10^{-7} M ATRA (−ATRA) and +ATRA, respectively) for 12 h. The signal was quantified using densitometric analysis of phosphorimaging data. PRAM-1 mRNA quantities were adjusted for GAPDH expression. In A (lower panel) and B (right panel), mean values and standard errors from two independent experiments are shown. C, PRAM-1 mRNA is induced in ATRA-treated primary APL cells. Cells were purified from three untreated patients cultivated in the absence (−) or presence (+) of 10^{-7} M ATRA for 5 (APL#2) or 6 (APL#1 and APL#3) days. Northern blots were performed using 8 μg (A and B) or 2 μg (C) of total RNA. Hybridization with a GAPDH probe controlled for RNA quantities in each lane.

**Fig. 2. Expression of PRAM-1 mRNA in primary hematopoietic tissues.** A and B, autoradiograms of PRAM-1 mRNA expression in immune tissues and peripheral blood cells. Northern blots were obtained from CLONTECH (A) or were performed using 5 μg of total RNA from granulocytes, monocytes, and lymphocytes (B). In A and B, GAPDH was used as a probe for assessment of RNA quantities in each lane. PBL, peripheral blood leukocytes. C, PRAM-1 mRNA is up-regulated during myeloid differentiation of human hematopoietic stem/progenitor cells. a, morphological differentiation of CD34+ cells toward the myeloid lineage. b, Southern blot analysis (upper panels) and relative expression (lower panel) of reverse transcripton-PCR products from CD34+ cells differentiating toward the myeloid lineage. Expression of β-actin mRNA was assessed as a control. Error bars indicate standard deviations from the results of three independent PCRs. This experiment was performed a second time with independent cell cultures, with similar results. A parallel PCR was conducted without addition of cDNA as a negative control (NC) or cDNA obtained from ATRA-treated NB4 cells as a positive control (PC).

**Fig. 3. PRAM-1 mRNA expression is dysregulated by PML-RARα.** A, PRAM-1 mRNA expression is down-regulated by ectopic expression of PML-RARα. In U937 cells and markedly increased following ATRA treatment. Autoradiogram (upper panels) and relative expression (lower panel) of the PRAM-1 mRNA in U937-PR9 and U937-MT cells either untreated (−) or treated (+) with 100 μM ZnSO4 for 12 h, followed by exposure to 5 × 10^{-7} M ATRA for different times. B, autoradiogram (left panels) and relative expression (right panel) of PRAM-1 mRNA in U937-PR9 and U937-MT cells treated with increasing concentrations of ZnSO4. Cells were either untreated or treated with 5 × 10^{-7} M ATRA (−ATRA) and +ATRA, respectively) for 12 h. The signal was quantified using densitometric analysis of phosphorimaging data. PRAM-1 mRNA quantities were adjusted for GAPDH expression. In A (lower panel) and B (right panel), mean values and standard errors from two independent experiments are shown. C, PRAM-1 mRNA is induced in ATRA-treated primary APL cells. Cells were purified from three untreated patients cultivated in the absence (−) or presence (+) of 10^{-7} M ATRA for 5 (APL#2) or 6 (APL#1 and APL#3) days.
PRAM-1, a Novel PML-RARα Target, Encodes an Adaptor Protein

We identified a novel adaptor protein, PRAM-1, which is induced by ATRA in human leukemia promyelocytes and is expressed and regulated during normal myelopoiesis. That PML-RARαs down-regulates expression of PRAM-1 mRNA in the absence of ligand indicates that PRAM-1 may be an oncogenic target of the fusion protein. Furthermore, superinduction of PRAM-1 by ATRA when PML-RARαs is expressed suggests that this adaptor may be an important component of a signaling pathway through which ATRA induces differentiation in leukemia promyelocytes.

Adaptor proteins have achieved increasing prominence in recent years because of recognition of their roles in numerous cellular processes (40–42). Much evidence supports the hypothesis that these proteins exert their effects upon differentiation through assembly of cytoplasmic signaling complexes that are required to modulate transcription. PRAM-1 shares homologies with SLAP-130/fyb, an adaptor protein initially newly diagnosed patients with APL (Fig. 3C) expressing the PML-RARα gene. This induction correlated with ATRA-induced differentiation of these cells, as assessed morphologically and by nitro blue tetrazolium reduction assay (data not shown).

**DISCUSSION**

We identified a novel adaptor protein, PRAM-1, which is induced by ATRA in human leukemia promyelocytes and is expressed and regulated during normal myelopoiesis. That PML-RARαs down-regulates expression of PRAM-1 mRNA in the absence of ligand indicates that PRAM-1 may be an oncogenic target of the fusion protein. Furthermore, superinduction of PRAM-1 by ATRA when PML-RARαs is expressed suggests that this adaptor may be an important component of a signaling pathway through which ATRA induces differentiation in leukemia promyelocytes.

**PRAM-1 Is an Adaptor Molecule—Using COS-7 cells transfected with a PRAM-1-expressing vector, anti-PRAM-1 polyclonal antibodies detected a specific protein migrating with an apparent molecular mass of 97 kDa (Fig. 4A, a). The discrepancy between the predicted molecular mass of 79 kDa and its migration on SDS-PAGE was probably due to numerous prolines and charged residues in PRAM-1, as also noted for the SLP-76 (37) and SLAP-130/fyb (21) adaptor proteins. In ATRA-treated NB4 cells, endogenous PRAM-1 migrated at the same rate as ectopically expressed PRAM-1 in COS-7 cells (Fig. 4A, a), and was tyrosine-phosphorylated upon pervanadate treatment (Fig. 4A, b). In kinetic studies, PRAM-1 protein expression was induced after 6 h of ATRA treatment and persisted through 6 days (Fig. 4B).

PRAM-1 shares sequence homologies with the adaptor protein SLAP-130/fyb, which can associate with SLP-76 (21, 22) and SKAP-HOM (also known as SKAP55-R) in T cells (29, 38).

**Proteins Involved in an ATRA-Signaling Pathway**

Upon finding that SLP-76 was expressed constitutively and that SLP-HOM was induced by ATRA in NB4 cells (Fig. 4B), we investigated whether PRAM-1 associates with these proteins. First, we documented the association of SLP-76 and SKAP-HOM with PRAM-1 by coexpressing PRAM-1 tagged with the F domain of the human estrogen receptor (F-PRAM-1) with Flag-tagged SLP-76 or Flag-tagged SKAP-HOM in COS-7 cells. Fig. 4C, a shows that in these cells Flag-SLP-76 and F-PRAM-1, as well as Flag-SKAP-HOM and F-PRAM-1 proteins, were detected in anti-F immunoprecipitates. We next asked whether PRAM-1 can associate with SKAP-HOM and SLP-76 in leukemic cell lines. As shown in Fig. 4C, b, PRAM-1 and SKAP-HOM from ATRA-treated NB4 cell lysates were detected in SKAP-HOM and PRAM-1 immunoprecipitates, respectively. We also observed that PRAM-1 was detected in SLP-76 immunoprecipitates, and SLP-76 was found in PRAM-1 immunoprecipitates from lysates of NB4 cells treated with both ATRA and pervanadate (Fig. 4C, c). Together, these data indicate that the PRAM-1 adaptor protein can associate with SLP-76 and SKAP-HOM in ATRA-treated NB4 cells. Because PTK have been shown to be induced in RA-treated HL-60 cells (39), we investigated whether PRAM-1 and lyn can associate in ATRA-treated NB4 cells. As shown in Fig. 4D, both PRAM-1 and lyn from ATRA-treated NB4 cell lysates were detected in PRAM-1 immunoprecipitate. Altogether, our results strongly suggest that PRAM-1 is an adaptor protein interacting with proteins involved in an ATRA-signaling pathway.

**Fig. 4. PRAM-1 is an adaptor molecule.** A, PRAM-1 protein is induced upon ATRA treatment in NB4 cells and is tyrosine-phosphorylated. a, COS-7 cells transfected with pMT-PRAM-1 were either untreated (−) or treated (+) with 75 μM ZnSO4 for 24 h. NB4 cells were treated with 10−8 M ATRA for 12 h. B, COS-7 cell lysates (10 μg) and protein extracts from 106 NB4 cells were separated by SDS-PAGE and immunoblotted for PRAM-1. b, NB4 cells were treated with 10−8 M ATRA for 72 h and either left unstimulated (−) or stimulated (+) with pervanadate (PV) for 5 min. Cells were then lysed, subjected to immunoprecipitation with anti-PRAM-1 antisera, and then assayed for phosphorytrosine (4G10) or PRAM-1. b, expression of PRAM-1, SKAP-HOM, and SLP-76 during ATRA-induced differentiation of NB4 cells. NB4 cells were treated with 5 × 10−8 M ATRA for different times. Protein extracts corresponding to 106 cells were separated by Western blot, using anti-PRAM-1, -SKAP-HOM, and -SLP-76 polyclonal antibodies. C, PRAM-1 associates with SKAP-HOM and SLP-76. a, vectors encoding F-PRAM-1, Flag-SLP-76, and Flag-SKAP-HOM were transfected (+) into COS-7 cells. 25-μg aliquots of each protein extract were immunoprecipitated (IP) with monoclonal anti-F antibodies. Immunoprecipitated proteins (right panels), as well as a 10-μg aliquot of the protein extracts (left panels) were separated by SDS-PAGE and analyzed for PRAM-1, SLP-76, and SKAP-HOM (Western blot, WB). b, protein extracts from ATRA-treated NB4 cells were immunoprecipitated (IP) with anti-PRAM-1 or anti-SKAP-HOM polyclonal antibodies. The arrowhead indicates the heavy chain of immunoglobulins (Ig H). c, ATRA-treated NB4 cells were either unstimulated (−) or stimulated (+) with pervanadate for 5 min. Lysates were subjected to immunoprecipitation with anti-SLP-76 or anti-PRAM-1 antisera and with the corresponding pre-immune serum. The resulting complexes were resolved by SDS-PAGE and analyzed for SLP-76 or PRAM-1. The asterisk indicates a nonspecific band. D, PRAM-1 associates with lyn in ATRA-treated NB4 cells. 120-μg aliquots of the ATRA-treated NB4 cell extracts were immunoprecipitated with anti-PRAM-1 polyclonal antibodies or with control PRAM-1 pre-immune serum (Control Ab). Immunoprecipitated proteins (right panels), as well as 40-μg aliquots of untreated or ATRA-treated protein extracts (left panels), were separated by SDS-PAGE and transferred onto polyvinylidene difluoride. Membranes were hybridized with anti-PRAM-1 or anti-lyn antibodies.
identified through associations with SLP-76 (22) and Lyn (21). The SLP-130 proline-rich region mediates binding to the SH3 domains of two related proteins, SKAP-HOM (38) and SKAP55 (43). Phosphorylated tyrosines amino-terminal to the SLP-130 SH3-like motif mediate binding to SLP-76 (44, 45). Similar domains within PRAM-1 may mediate the association between PRAM-1 and SLP-76 or PRAM-1 and SKAP-HOM. Given the observations of shared structure and binding partners for PRAM-1 and SLP-130, the two molecules may occupy a similar functional niche. Myeloid cells express SLP-130 (21), and several observations suggest that it plays a role in myeloid signal transduction through Fc receptors (46) by coupling Fc receptor-stimulated protein tyrosine kinases to downstream signaling events. Furthermore, in murine bone marrow-derived macrophages, SLP-130 is phosphorylated and inducibly associated with SLP-76 after exposure to antibody-coated sheep red blood cells. Our data showing that PRAM-1 is structurally similar to SLP-130 and that it inducibly associates with SLP-76 thus reinforces the view that it may also regulate signaling through Fc receptors. SKAP-HOM, which can associate with PTK Lyn (29), forms a complex with SLP-130, which associates with the macrophage inhibitory receptor SHPS-1 (47). Our observation that PRAM-1 associates with SKAP-HOM raises the possibility that PRAM-1 might also participate in signaling through additional myeloid receptors. Furthermore, SKAP-HOM can associate with PTK Lyn (48), which is induced and tyrosine-phosphorylated in RA-treated myeloid leukemia cells. Coordinate induction of PRAM-1, SKAP-HOM, and lyn protein expression by ATRA in NB4 cells, coupled with capacity of the two adaptors to associate together and with lyn, suggest that PRAM-1, SKAP-HOM, and lyn may be functionally linked in a common ATRA-dependent signaling pathway. The PTK Lyn prevents apoptosis to promote RA-induced differentiation of HL-60 cells (39). Whether PRAM-1 is involved in signaling through Fc receptors, SKAP-HOM, which can associate with PTK Lyn (48), may mediate the association between the two adaptors to associate together and with lyn, thereby freeing pre-leukemic cells from controls that normally induce the onset of differentiation. This, in association with other genetic dysregulations, may contribute to full leukemic transformation. It is also likely that treatment of APL cells by ATRA induces de novo up-regulation of the same genes that are dominantly repressed by PML-RARα and whose expression is required for reactivation of the differentiation program (16). To date, the myeloid transcription factor CCAAT-enhancer-binding protein ε is the only example of such a gene (49). PRAM-1 is a first example of an adaptor molecule whose expression is inhibited and induced superinduced when PML-RARα is expressed alone and in the presence of ATRA, respectively. The evidence that PRAM-1 mRNA expression is inhibited by PML-RARα, together with the association of PRAM-1 with other RA-induced proteins including lyn, suggest that the fusion protein may indirectly dysregulate the organization of molecular complexes comprising scaffold proteins and therefore disrupt signaling events important for ATRA-induced differentiation.

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PRAM-1 Is a Novel Adaptor Protein Regulated by Retinoic Acid (RA) and Promyelocytic Leukemia (PML)-RA Receptor α in Acute Promyelocytic Leukemia Cells

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