The retinoic acid-responsive proline-rich protein is identified in promyeloleukemic HL-60 cells

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To identify new genes that retinoic acid activates, we employed an mRNA differential display technique and screened for genes that are differentially expressed in promyeloleukemic HL-60 cells incubated in the presence of all-trans-retinoic acid (ATRA) compared with the absence of ATRA. We cloned the coding region of a retinoic acid-induced gene from a human thymus library, which was the mRNA encoding the 666-amino acid human homologue of mouse proline-rich protein 76. We have designated it RARP1 (retinoic acid response proline-rich protein 1). Transcription of an ~2.4-kbp mRNA occurred mainly in organs with immune functions, such as thymus, spleen, and peripheral leukocytes. Cycloheximide blocked the ATRA-induced expression. In megakaryocyte-like human erythroleukemia HEL cells, the amount of RARP1 mRNA was high but was low in human T-lymphoblastoid Jurkat cells. A specific antibody against RARP1 recognized a 110-kDa protein, which accumulates after incubation of HL-60 cells with ATRA. In immunohistochemical experiments, strong RARP1 staining was observed in the megakaryocytes of bone marrow and spleen, and heterogeneous stain was seen in thymus. Transcriptional studies showed that RARP1 expression impaired the transactivation through activator protein1 and serum response element in all cell lines we checked, whereas it did not affect the transactivation through cAMP-response element in the same cell lines. Further analysis demonstrated that proline-rich regions of RARP1 are the functional regions regulated for suppression of activator protein1 transactivation. These data suggest that ATRA-inducible RARP1 selectively affects signal transduction and may contribute to myeloid and megakaryocytic differentiation.

Retinooids and their active metabolites, including all-trans-retinoic acid (ATRA), play an important role in morphogene-
sis, differentiation, and homeostasis (1). In the hematopoietic system, Breitman et al. (2) first reported the action of ATRA to promote HL-60 cell differentiation in vitro. The specific action has led to widespread use of ATRA to promote induction of neutrophilic differentiation of leukemia cells as a selective means of chemotherapy for fresh acute promyeloleukemic cells (3). Retinoic acid receptors and retinoic X-receptors are two distinct families of nuclear receptors that belong to the steroid/thyroid hormone receptor family (4). ATRA binds with retinoic acid receptors, and another retinoid, 9-cis-retinoic acid, binds with retinoic X-receptors (5). DNA array, suppression-subtractive hybridization, and differential display-PCR analyses demonstrated that some genes are up- and down-regulated directly or indirectly by the treatment with ATRA in human promyeloleukemia NB4 cells (6). Thus, it is understandable that retinoic acid directly or indirectly activates a series of genes that may contribute to differentiation of HL-60 cells. To identify the gene that responds to retinoic acid, we employed a differential display technique using the mRNA from HL-60 cells treated with or without ATRA. We cloned the human homologue of "mouse proline-rich protein 76" and evaluated the mRNA expression in various tissues and reactivity to other differentiation-induced agents. The mRNA was preferentially expressed in the hematopoietic and immune systems, and the marked protein expression was observed in megakaryocytes. Because the protein contains putative Ras-associated domain, pleckstrin homology (PH) domain, and proline-rich domain, we evaluated the effect of the RARP1 expression on signal transduction in vitro.

EXPERIMENTAL PROCEDURES

Cell Culture and Isolation of Poly(A) RNA—HL-60 and Jurkat cells were purchased from RIKEN. The cells were cultured with RPMI media containing 10% fetal bovine serum. CHO-K1 and CHO-IR cells were cultured as described previously (7). Total RNA was isolated from HL-60 cells after 24 h of incubation in the presence or absence of 1 μM ATRA using RNasey mini kit (Qiagen Inc., Valencia, CA). Poly(A) RNA was purified by QuickPrep Micro mRNA purification kit (Amersham Biosciences).

mRNA Differential Display—Differential display PCR was performed using Genehunter RNA image kit (Genehunter Corp., Nashville, TN), according to the manufacturer’s protocol. Two hundred ng of poly(A) RNAs from treated cells were reverse-transcribed with 100 units of Moloney murine leukemia virus-reverse transcriptase in the presence of one-base anchored oligod(T) primers for 1 h at 37°C in a total volume of 20 μl. The reaction was terminated by incubation at 75°C for 5 min. Two μl of the reaction mixture was PCR-amplified with AmpliTaq (Applied Biosystems, Foster City, CA) in 0.2 μM HA-F4 (5’-AAGCTTTGCAAGG-3’), 13-mer (5’-end primers), and oligog(T)15 primers (5’-AGGTTCACGTTT-3’) (3’-end primers), using a Gene

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Amp PCR system 2400 (PerkinElmer Life Sciences). After cloning into the pGEM-T Easy vector using the TA cloning system (Promega, Madison, WI), the sequenced cDNAs were analyzed via the BLAST program for matches in the GenBank/EMBL/DBJ data base and were compared with each other via FASTA analysis (DBJB, Mishima, Japan). The motif search was carried out using Interpro Scan (www.ebi.ac.uk/interpro/scan.html).

Cloning and Northern Hybridization Analysis—PCR-reamplified cDNA fragments were subcloned into pGEM-T easy vector (Promega, Madison, WI). Differential expression of the cDNA fragment in the presence or absence of ATRA was confirmed by Northern hybridization following standard procedures. The differentially expressed cDNA fragment or the fragment including the full coding region, labeled with a [α-32P]dCTP by random prime labeling, was used as the probe for hybridization. As a loading control, elongation factor 1α (EF1) mRNA was detected with an EF1 cDNA probe as described previously (8). For the experiment with cycloheximide (Fig. 5c), HL-60 cells were treated for 12 h with medium alone, 1 μM ATRA, 10 μg/ml cycloheximide, or 1 μM ATRA plus 10 μg/ml cycloheximide. For the experiment with HL-60, HEL, and Jurkat cells (Fig. 5d), each cells were treated for 24 h with or without 1 μM ATRA. For the experiment with several compounds (Fig. 5e), HL-60 cells were treated for 24 h with 0.1% dimethyl sulfoxide, 10 mM 12-O-tetradecanoylphorbol-13-acetate (TPA), 10 mM trioglate, 1 μg 1,25-dihydroxyvitamin D₃, or 1 μM ATRA. Total RNA was isolated from HL-60 cells using RNeasy mini kit (Qiagen Inc., Valencia, CA).

Construction of the Mammalian Expression Vector and Its Deleted Protein Expression Vectors—The EcoRI-BlnI fragment was cloned into EcoRI-XbaI site of pCDNA3.1 vector (Invitrogen). The BlnI fragment including complete coding region of the RARP1 was cloned into the mammalian expression vector (Clontech Laboratories (Palo Alto, CA). 2234-bp fragment was isolated from the library. The 5′-end of the gene was cloned by using 5′-RACE method. The 5′-end and 3′-fragment were subcloned into pEG32f. The two clones were digested with BgIII restriction site presented in the overlapping region, and the resulting fragments were ligated to obtain full-length RARP1 cDNA. Both the forward and the reverse strands of the cDNA were sequenced to determine the complete cDNA sequence.

Preparation of Polyclonal Antibodies to RARP1—Synthetic peptides containing a part of the RARP1 amino acid sequence were used in the immunization. The amino acid sequence (CPVPPKRQENPHPGQ) was chosen because of its high antigenicity index, determined by Epitope Adviser (Fujitsu, Shizuoka, Japan). The synthetic peptide was chosen because of its high antigenicity index, determined by Epitope Adviser (Fujitsu, Shizuoka, Japan). The synthetic peptide was conjugated to Keyhole limpet hemocyanin (KLH) and used for immunization with rabbits and were purified by affinity column chromatography with the immunized peptide.

Preparation of Differentially Expressed Fragments—We performed differential display PCR method. The poly(A) RNAs from HL-60 cells treated with indicated ligands for 24 h were reverse-transcribed in the presence of one-base anchored oligo(dT) primers. The reaction mixture was PCR-amplified with H-AP4′ (5′-AAGCCTCTTCAACG-3′), a 13-mer (5′-end primers), and oligo(dT)₁₁, primers (5′-AAGCCTTTTTTTTTTTTTG-3′) (5′-end primers) in the presence of α-32P-dATP. The sample was applied on 6% sequencing gel and exposed for 5 days. The arrow indicates an ~340-bp fragment was enhanced by the treatment with ATRA.

RESULTS

Identification of Differentially Expressed Fragments—We performed differential display on the mRNA from HL-60 cells treated with and without 1 μM retinoic acid (Fig. 1). One of the differentially expressed cDNA fragments, named AP4G, was re-amplified from the band and cloned into pGEM-T vector. The resultant clone was sequenced. Sequence analysis showed that the sequence of AP4G must be located on the 3′-end of a novel gene with unknown function.

Cloning the Full-length cDNA of RARP1—Screening of the cDNA fragments was performed as described previously (9). Briefly, cells were lysed by adding lysis buffer (0.6% SDS, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100) and were resuspended in wash buffer containing 2% SDS and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked TBS-T (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20) containing 5% skim milk. Detection was carried out by using the enhanced chemiluminescence using a horseradish peroxidase-coupled goat anti-rabbit IgG antibody (Amersham Biosciences). Six-week-old mouse frozen tissues were cut in 20-μm sections using a cryostat. After blocking with 1% bovine serum albumin in PBS, sections were reacted with the primary antibody (1:250 dilution) 1 h at room temperature. After washing with PBS, sections were incubated with FITC-labeled swine anti-rabbit IgG (1:100 dilution) (Dako, Carpinteria, CA) for 2 h at room temperature. In double staining experiments, sections were reacted with rabbit anti-RARP1 antibody and rat anti-CD41 antibody for 1 h at room temperature. After washing with PBS, sections were incubated with TRITC-labeled swine anti-rabbit IgG and FITC-labeled swine anti-rat IgG for 2 h at room temperature as the second antibody. After washing with PBS, the sections were mounted with Slow-Fade (Molecular Probes, Portland, OR). Cells expressing FITC and TRITC were imaged on a FUJUVIEW laser-scanning confocal microscope mounted on a BX-50 inverted fluorescence microscope equipped with a 20 × 0.5 N.A. lens (Fig. 6, c-e) or a 40 × 1.0 N.A. oil immersion lens (Fig. 6f).

Promoter-Reporter Assay—To detect the pathway activated in the presence of RARP1, we utilized the Mercury Pathway Profiling Systems (Clontech, Palo Alto, CA). Briefly, CHO-K1, Jurkat, and CHO-IR cells were cultured in 24-well dishes and transiently transfected with promoter-reporter plasmids using LipofectAMINE (Invitrogen) with serum-free Ham’s F-12 media as described in the manufacturer’s protocol. These plasmids contained the luciferase reporter gene downstream of several copies of specific transcription factors binding sequences such as AP-1, cyclic AMP-response element (CRE), and serum-response element (SRE). In addition, we cotransfected with βSV-β-galactosidase vector (Promega Corp., Madison) in the presence or absence of RARP1 expression vector. Transfected cells were allowed to recover for 24 h after incubating with or without 100 ng/ml TPA, 5 μg dibutyryl cAMP, and 5% fetal bovine serum, respectively. Luciferase activity was determined by the Luciferase Assay System (Promega Corp., Madison) according to the protocol using Berthold Lumat (EG&G Berthold, Eyrv, France). The activity of β-galactosidase was measured by the method described previously (10), and all luciferase data were corrected for β-galactosidase activity to account for variations in transfection efficacy.

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to screen a normal thymus cDNA library. One of the positive clones containing a 2.2-kb (clone 9) insertion was sequenced. By searching with FASTA program, we identified two sequences (GenBankTM/EMBL/DDBJ accession numbers AK000812 and BC023110). 

AK000812 is a human gene whose nucleotide 458–839 was completely the same as the 5'-end of clone 9. BC023110 was a mouse clone that denotes "proline-rich protein 76" whose function is not known. Based on this information, we carried out 5'-RACE. Assembly of the clones and the RACE-PCR sequences gives 2456 nucleotides. The cDNA sequence contains an open reading frame encoding a polypeptide of 666 amino acids (Fig. 2). Protein homology search with Interpro Scan predicted that there were three functional domains, such as the proline-rich domain, the Ras-associated domain, and the PH domain. The identity score between mouse proline-rich protein 76 and RARP1 was 79.5%. There were two highly homologous regions. One was 83 amino acids in N-terminal region (96.4%). The other was the middle region between 158 alanine and 446 valine including the Ras-associated domain and the PH domain (94.8%). Although the similarities were low in the two proline-rich regions between 84 leucine and 157 glutamine, and 223 amino acids in C-terminal region, the localization of proline residues was conserved. The complete nucleotide sequence of the RARP1 cDNA has been deposited into the DDBJ/GenBankTM/EBI Data Bank as accession number AB085852.

Tissue Distributions of the RARP1—Analyses of the tissue distributions of RARP1 transcript were carried out using Northern blot membranes manufactured in Clontech. A single band whose length is 2.6 kb was identified (Fig. 3). RARP1 was dominantly expressed in thymus, spleen, liver, and peripheral leukocytes.

Characterization of RARP1 Expression—The RARP1 was induced by ATRA in a dose-dependent manner (Fig. 4a). Northern analysis showed that the mRNA was increased 6–12 h after adding 1 μM ATRA in HL-60 cells (Fig. 4b). The treatment with cycloheximide suppressed the expression of the elongation factor 1α (EF1) in HL-60 cells (Fig. 4e, middle panel). The expression of RARP1 was slightly affected by cycloheximide (Fig. 4e, upper panel), although the treatment with cycloheximide blocked the induction of RARP1 with ATRA. The expression in HEL cells was higher than that in HL-60 cells and was increased by the treatment with ATRA, whereas the expression was low and did not respond in Jurkat cells (Fig. 4d). ATRA increased the RARP1 mRNA, although the other known differentiation agents studied did not induce the mRNA (Fig. 4e).

RAR1 Protein Accumulates in HL-60 Cells after Incubation with ATRA—We raised specific antibody against RARP1 peptide. A 110-kDa protein was detected in the HL-60 cell lysate treated with 1 μM ATRA for 24 h (Fig. 5a). The expression of the protein was accumulated during 5 days of incubation with 1 μM ATRA in HL-60 cells. The molecular mass of the protein was similar to that expressed in CHO-K1 cells transfected with the mammalian expression vector for RARP1 (Fig. 5b). The cytoplasm was dominantly stained with anti-RARP1 antibody (Fig. 5c).

Immunohistochemical Findings—Because mRNA expression
was dominant in the tissues associated with immune and hematopoietic systems, we performed immunohistochemistry by using the antibody in thymus, spleen, and bone marrow. Heterogeneous staining was observed in thymus (Fig. 5d). Marked expression was detected in the cytoplasms of megakaryocytes or their progenitor cells whose surface was stained with FITC-labeled CD41 antibody in bone marrow and spleen (Fig. 5, e–g).

**RARP1 Suppresses TPA- or SRE-driven Transcription**—Because motif scanning analyses showed putative Ras-associated domain, PH domain, and proline-rich domain in the RARP1 molecule, we initially evaluated the function of the protein as signaling mediators. To detect the signaling pathway activated in the presence of RARP1 expression, we utilized the Mercury Pathway Profiling Systems (Clontech, Palo Alto, CA). Several cis-acting enhancer elements, such as AP1 (activator protein1), CRE, and SRE included in this system, were assessed in CHO-K1, Jurkat cells exposed to 100 ng/ml TPA, 5 mM dibutyryl cAMP, and 5% fetal bovine serum, respectively (Fig. 6, a and b). Transcription from the CRE element was not affected, whereas those from the AP1 and SRE were found to be inhibited by the expression of RARP1 in the cell lines studied. To demonstrate the selective effect of RARP1 on endogenous pathways of the signal transduction more clearly, we used CHO-IR cells that possess permanently overexpressed insulin receptor (7). Insulin increased transcriptional activity through AP1, SRE, as well as CRE in the cell line. Expression of RARP1 diminished insulin-induced promoter activities through AP1 and SRE but not through CRE (Fig. 6c).

**Two Proline-rich Domains Were Required for Suppression of Insulin-induced Transactivity through AP1**—To map the functional regions for suppression of AP1 transactivation, we transfected a series of deletion mutants of RARP1 in CHO-IR and evaluated transactivity after stimulation with insulin. As shown in Fig. 7, the insulin-induced AP1 activity was reduced by 23% in the presence of the intact RARP1. The region between 1 and 124 did not affect the transactivity, whereas the region between 1 and 176 decreased the activity by 65%. The suppression was not observed when we transfected the region between 175 and 419, whereas the region between 175 and 666 inhibited the AP1 transactivity by 54%.

**Fig. 4. Characterization of the expression of the RARP1 mRNA.** a, total RNAs were extracted from HL-60 cells treated with 0.1% ethanol and 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M ATRA for 24 h. Ten μg of the RNA was applied onto the gel and transferred to a membrane. Northern hybridizations were performed with the labeled RARP1 (upper panel) and rat elongation factor1a (EF1) (lower panel) individually. b, total RNAs were extracted from HL-60 cells treated without ATRA and with ATRA for 3, 6, 12, and 24 h. Ten μg of the RNA was applied on the gel and transferred to a membrane. Northern hybridizations were performed with the labeled RARP1 (upper panel) and EF1 (lower panel) individually. c, HL-60 cells were treated for 12 h with medium alone, 1 μM ATRA, 10 μg/ml cycloheximide (CHX), or 1 μM ATRA plus 10 μg/ml cycloheximide. Thirty μg of total RNA was transferred to a membrane. Northern hybridizations were performed with the labeled RARP1 (upper panel) and EF1 (middle panel) individually. Ethidium bromide staining of the gel was shown (lower panel). d, total RNAs were extracted from HL-60, human erythroleukemia HEL, and human T-cell lymphoma Jurkat cells in the presence or absence of 10⁻⁶ M ATRA for 24 h. Ten μg of the RNA was transferred to a membrane. The membrane was hybridized with labeled RARP1 fragment (upper panel) and EF1 fragment (lower panel) individually. e, ATRA induced the expression of the RARP1, whereas other agents studied did not in HL-60 cells. HL-60 cells were treated with the indicated ligands for 24 h. The total RNAs were extracted and transferred to a membrane. Northern hybridization was performed with labeled RARP1 (upper panel) and EF1 (lower panel) individually. DMSO, dimethyl sulfoxide; TGD, troglitazone; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃.
We have isolated a novel ATRA-response gene by using differential display technique. The FASTA analysis demonstrated that the gene was a human homologue of mouse proline-rich protein 76. The protein was designated RARP1. Structural analysis demonstrated that the protein contains a putative Ras-associated domain, a PH domain, and two proline-rich domains. Ras-associated domain is reported as the effector molecule for R-Ras, K-Ras, and Rap (11). The function of the PH domain is not clear, but this domain binds to heterotrimeric G proteins, lipids such as phosphatidylinositol 4,5-bisphosphate, and phosphorylated serine/threonine residues (12). The proline-rich domain is a low complexity region that is found in a variety of proteins from prokaryotes and eukaryotes. It is reported as the molecule of the effector domain for Src homology domain 3 (SH3) (13). These bioinformatic data suggest that the gene product, at least in part, may be related to the signaling mechanisms. There are numerous ATRA up- and down-regulated genes that are associated with signal transduction, such as fgr, protein kinase Ca, inositol triphosphate receptor, c-fms, and Srr-like adapter protein blr-1 (14–19). The ATRA-induced alteration of the expression of these genes including the RARP1, which might induce a different response to the extra-cellular signals, results in the differentiation of the cells.

The similarity to the mouse homologue showed that 83 amino residues in the N-terminal region, the Ras-associated domain, the PH domain, and the position of proline residues in the two proline-rich domains are conserved, suggesting that these regions are crucial for the physiological function of the molecule.

The RARP1 mRNAs are preferentially expressed in thymus, spleen, liver, and peripheral leukocytes. Because immunohistochemically liver was not stained with anti-RARP1 antibody (data not shown), the mRNA signal in liver was probably derived from the contaminated leukocytes or lymphocytes. Thus, the expression may be overestimated in liver. The distribution of the RARP1 mRNA suggests that the protein might be related to the hematopoietic and immune function.

The RARP1 induction was detected 6–12 h after incubation with ATRA. It is consistent with most of the ATRA-activated genes induced 12–48 h after treatment with ATRA in promyeloleukemic cells (6). Cycloheximide suppressed the expression of EF1 as demonstrated previously (20). The constitutive expression of RARP1 was slightly affected by the treatment with cycloheximide, suggesting that ATRA-induced proteins are required for ATRA-induced expression of RARP1 at the transcriptional or post-transcriptional level. The higher expression was observed in HEL cells, whereas the lower expression was observed in Jurkat cells.
FIG. 6. a and b, RARP1 did not affect cAMP-induced transcriptional activity through the cAMP-response element and blocked serum- and TPA-induced transcriptional activity through the serum-response element and AP1, respectively in CHO-K1 (a) and Jurkat cells (b). Transient cotransfection experiments in CHO-K1 cells (a) and Jurkat cells (b) with the SRE, AP1, and CRE hooked thymidine kinase (TAL) promoter construct in the presence or absence of 5% serum, 100 ng/ml TPA, and 5 mM dibutyric cAMP, respectively. Cotransfection with the TAL promoter
indicating various expression levels in leukemia and lymphoma cells.

Various stimulators are reported to induce the differentiation in HL-60 cells (21). Although dimethyl sulfoxide (Me₂SO), TPA, thiazolidinedione including troglitazone, 1,25 (OH)₂D₃ as well as ATRA induce differentiation in HL-60 cells, the RARP1 expression was not induced by the compounds except ATRA. The data suggest that the retinoic acid is a potent initiator for

**Fig. 7.** Proline-rich domain was required for the repression of insulin-induced transactivity through AP1 in CHO-IR cells. a, schematic representation of RARP1 protein. b, transient transfection with AP1 promoter construct and the indicated intact RARP1 (RARP1-w) or the mutant RARP1 expression vectors in the presence or absence of insulin in CHO-IR cells. Twenty hours after the stimulation, cells were harvested and measured for the luciferase and β-galactosidase activities. All luciferase activities were corrected for β-galactosidase activities. Each value represents the mean ± S.D. of four separate determinations. Asterisk indicated statistical significance to the activity obtained in the absence of RARP1 or RARP1 mutants (p < 0.01). N.S., no significance.

| Construct        | INS(-) | INS(+) |
|------------------|--------|--------|
| pcDNA            | 2500   | 1500   |
| RARP1-w          | 1200   | 900    |
| RARP1-124        | 750    | 450    |
| RARP1-176        | 500    | 250    |
| RARP175-419      | 375    | 225    |
| RARP175-666      | 300    | 150    |

indicating various expression levels in leukemia and lymphoma cells.
the induction of RARP1 in HL-60 cells.

On the other hand, the protein levels gradually increased after 5 days of incubation with ATRA. ATRA induces apoptosis in HL-60 cells. Since the numbers of the surviving cells decreased during the 5-day incubation with ATRA, the extracts of the protein were mainly obtained from surviving cells, suggesting that the RARP1 expression may be limited to intracellular regions in surviving and/or differentiated cells.

Heterogeneous expression of the RARP1 protein in thymus suggests that the expression of the protein may relate to the T-cell development. Marked expression in megakaryocytes implies that the function of the protein relates to megakaryocyte differentiation.

The molecular mechanisms of RARP1 action are as yet unknown. The gene expression profiling study demonstrated that ATRA induces or suppresses the genes that are participating in the signal transduction pathways including JAKs/STAT, cAMP/protein kinase A, protein kinase C, and mitogen-activated protein kinase in NB4 cells (6). The presence of several protein domains that are characteristic for signal regulators suggests that RARP1 can directly or indirectly modulate signal transduction. This hypothesis is supported by the selective inhibitory effect of the protein on the transcriptional activity through AP-1 and SRE and not CRE. The two proline-rich domains were required for the suppressive effect of AP1 signaling in the molecule, indicating that the domain-specific function may be present in the molecule of the RARP1.

In summary, we have identified RARP1 as the ATRA-induced product in HL-60 cells. Its induction was specifically controlled by ATRA. The protein expression affected signaling mechanisms selectively through its proline-rich domains. Because RARP1 expression was also expressed at high levels in megakaryocyte as well as thymus in vivo, it will be important to determine whether it affects not only myeloid differentiation but also cellular development and/or disease in the lymphoid and megakaryotic lineage of the cells.

REFERENCES

1. Lotan, R. (1980) Biochim. Biophys. Acta 605, 33–91
2. Breitman, T. R., Selonick, S. E., and Collins, S. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2956–2960
3. Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhao, L., Gu, L. J., and Wang, Z. Y. (1988) Blood 72, 567–572
4. Pfahl, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M. O., La-Vista, N., Lu, Y. P., Piedraflita, J., and Ortiz, M. A. (1994) Vitam. Horm. 49, 327–382
5. Levin, A. A., Sturzenbecker, J. L., Kazmer, S., Bosakowski, T., Huseulton, C., Allenby, G., Speck, J., Kratzteizen, C., Rosenberger, M., Loverly, A., and Gripp, J. F. (1992) Nature 355, 359–361
6. Liu, T.-X., Zhang, J.-W., Tao, J., Zhang, R.-B., Zhang, Q.-H., Zhao, C.-J., Tong, J.-H., Lanotte, M., Waxman, S., Chen, S.-J., Mao, M., Hu, G.-X., and Chen, Z. (2000) Blood 96, 1496–1504
7. Yamauchi, K., and Pessin, J. E. (1994) Mol. Cell. Biol. 14, 4427–4434
8. Ann, D. K., Lin, H. H., Lee, S., Tu, Z.-J., and Wang, E. (1992) J. Biol. Chem. 267, 699–702
9. Suzuki, S., Mori, J., Kobayashi, M., Inagaki, T., Inaba, H., Komatsu, A., Yamashita, K., Takeda, T., Miyamoto, T., Ichikawa, K., and Hashizume, K. (2003) Eur. J. Endocrinol. 148, 259–268
10. Mori, J., Suzuki, S., Kobayashi, M., Inagaki, T., Komatsu, A., Takeda, T., Miyamoto, T., Ichikawa, K., and Hashizume, K. (2002) Endocrinology 143, 1528–1544
11. Hofer, F., Fieldis, S., Schneider, C., and Martin, G. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11089–11093
12. Saraste, M., and Hyvonen, M. (1995) Curr. Opin. Struct. Biol. 5, 403–408
13. Pessin, J. E., and Okada, S. (1997) Endocr. J. (Suppl.) 46, S11–S16
14. Katagiri, K., Katagiri, T., Koyama, Y., Morikawa, M., Yamamoto, T., and Yoshida, T. (1991) J. Immunol. 146, 701–707
15. Devalia, V., Thomas, N. S., Roberts, P. J., Jones, H. M., and Linch, D. C. (1992) Blood 80, 68–76
16. Bradford, P. G., Wang, X., Jin, Y., and Hui, P. (1992) J. Biol. Chem. 267, 20953–20964
17. Hsu, H. C., Yang, K., Kharbanda, S., Clinton, S., Datta, R., and Stone, R. M. (1993) Leukemia (Baltimore) 7, 458–462
18. Ohita, T., Hatake, K., Ikeda, M., Tomizuka, H., Terui, Y., Uwai, M., and Miura, Y. (1997) Biochem. Biophys. Res. Commun. 230, 81–84
19. Battle, T. E., Roberson, M. S., Zhang, T., Varvayanis, S., and Yen, A. (2001) Eur. J. Cell Biol. 80, 59–67
20. Chacko, G., Ling, Q., and Hajjar, K. A. (1998) J. Biol. Chem. 273, 19840–19846
21. Breitman, T. R., Hemmi, H., and Inaizumi, M. (1986) Proc. Clin. Biol. Res. 226, 215–233