Protein kinase C\(\beta\) mediates retinoic acid and phorbol myristate acetate–induced phospholipid scramblase 1 gene expression: its role in leukemic cell differentiation

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Although phospholipid scramblase 1 (PLSCR1) was originally identified based on its capacity to promote transbilayer movement of membrane phospholipids, subsequent studies also provided evidence for its role in cell proliferation, maturation, and apoptosis. In this report, we investigate the potential role of PLSCR1 in leukemic cell differentiation. We show that all-trans retinoic acid (ATRA), an effective differentiation-inducing agent of acute promyelocytic leukemia (APL) cells, can elevate PLSCR1 expression in ATRA-sensitive APL cells NB4 and HL60, but not in maturation-resistant NB4-LR1 cells. ATRA- and phorbol 12-myristate 13-acetate (PMA)–induced monocytic differentiation is accompanied by increased PLSCR1 expression, whereas only a slight or no elevation of PLSCR1 expression is observed in U937 cells differentiated with dimethyl sulfoxide (DMSO), sodium butyrate, or vitamin D3. Cell differentiation with ATRA and PMA, but not with vitamin D3 or DMSO, results in phosphorylation of protein kinase C\(\beta\) (PKC\(\beta\)), and the PKC\(\beta\)-specific inhibitor rottlerin nearly eliminates the ATRA- and PMA-induced expression of PLSCR1, while ectopic expression of a constitutively active form of PKC\(\beta\) directly increases PLSCR1 expression. Finally, decreasing PLSCR1 expression with small interfering RNA inhibits ATRA/PMA-induced differentiation. Taken together, these results suggest that as a protein induced upon PKC\(\beta\) activation, PLSCR1 is required for ATRA- and PMA–triggered leukemic cell differentiation.

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terminal differentiation into neutrophils and macrophages. Conversely, de novo expression of a mutant mRNA encoding a truncated form of murine PLSCR1 (also known as MmTRA1a, deleting the proline-rich segment between codons 1-128) was identified in a monocytic leukemia cell line, and this mutation was found to correlate with the ability of these cells to proliferate in vivo. By contrast, the expression of full-length PLSCR1 induced differentiation of these leukemic cells to macrophages. Furthermore, Nakamaki et al. reported that PLSCR1 mRNA was specifically induced during granulocytic differentiation of acute promyelocytic leukemia (APL) cells by all-trans retinoic acid (ATRA), a widely studied potent inducer of cell differentiation and growth arrest of malignant cells in vitro and in vivo. Finally, a recent study performed in patients with acute myelogenous leukemia (AML) showed that higher levels of PLSCR1 mRNA were associated with significantly longer overall survival, particularly in patients of the AML-M4 subtype, independent of chromosomal aberrations such as t(8;21) and inv(16), suggesting PLSCR1 mRNA level as a new prognostic factor for AML.

In this study, we investigate effects of known differentiation-inducing agents on PLSCR1 expression in leukemic cell lines in order to gain insight into a potential role of PLSCR1 in leukemic cell differentiation. We show that PLSCR1 is significantly up-regulated during both granulocytic and monocytic differentiation induced by ATRA and phorbol 12-myristate 13-acetate (PMA), and that activation of PKCδ is required for this process. Furthermore, by ectopic expression of a constitutively active form of PKCδ, we demonstrate for the first time that PKCδ can directly induce PLSCR1 expression. Finally, using small interfering RNA (siRNA) to decrease cellular PLSCR1 expression, we provide evidence that PLSCR1 is required for ATRA- and PMA-induced leukemic cell differentiation.

Materials and methods

Cells and cell treatment

Leukemia cell lines used in this study included chromosomal translocation t(15;17)-positive and ATRA-sensitive human APL cell line NB4 and NB4-derived maturation-resistant, ATRA-responsive cell line NB4-LR1; t(15;17)-negative ATRA-sensitive APL cell line HL60; human acute monocytic leukemia cell line U937; and human T-lymphocytic leukemia cell line Jurkat. In addition, adherent cell line Cos-7 was also used. All cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) and were treated with the indicated concentrations of ATRA, PMA, 12-O-tetradecanoylphorbol-13-acetate (TPA), or 1-25-hydroxyvitamin D3 (1,25(OH)2D3). Cells and cell treatment

Plasmids and transient transfection

Plasmids pEGFP-N1 and pEGFP-PKC-CF6 carrying the catalytic fragment of PKCδ were obtained as a generous gift from Dr Mary E. Reylend (Denver, CO). These plasmids were transfected into Cos-7 cells using Polyfect transfection reagent (QiAGEN, Valencia, CA) according to the manufacturer’s instructions. pEGFP-N1 plasmid was transfected as a negative control. Transfected cells were analyzed 48 hours after transfection for expression of PLSCR1.

siRNA design and stable expression

The mammalian expression vector pSiSilencer 3.1-H1 neo (Ambion, Austin, TX) was used for expression of siRNA in U937 cells. siRNAs to PLSCR1 were designed following the procedure from Ambion. There were 5 target sequences selected: P1, 5'-TCA GCC AGT ATA TAA TCA G-3'; P2, 5'-CTC TGG AGA GAC CAC TAA G-3'; P3, 5'-ATA AGT GGT CCA TGT GTT G-3'; P4, 5'-TTT CCA AGC ACT GGA CTG G-3'; and P5, 5'-AGT CTC CTC AGG AAA TCT G-3'. Each sequence was aligned to the human genome database in a BLAST search to eliminate those with significant homology to other genes. For each target sequence, we designed complementary 55- to 60-mer oligonucleotides with 5' single-stranded overhangs for ligation into the pSilencer 3.1-H1 neo vector. The oligonucleotides encoded 19-mer hairpin sequences specific to PLSCR1 mRNA target, a loop sequence separating the 2 complementary domains, and a polythymide tract to terminate transcription. These sequences were synthesized and inserted into the pSilencer 3.1-H1 neo vector according to the manufacturer’s instructions (Ambion). U937 cells were transfected using Nucleofector Solution (Amazza, Gaithersburg, MD) according to the manufacturer’s instructions. At 48 hours after transfection, 800 μg/mL G418 was added to the medium to select the stable transfected cells.

Evaluation of cell differentiation

Cell differentiation was evaluated by morphologic characterization and the percentage of mature-related cell-surface differentiation antigens CD11b, CD11c, and CD14. For morphologic observation, cells were collected onto slides by cytospin (Shandon, Runcorn, United Kingdom), stained (Wright staining), and observed by light microscope (Olympus, B×51, Tokyo, Japan). The images were captured with Olympus DP50 digital camera by Image-Pro plus. Differentiation antigens were measured by flow cytometry (Beckman-Coulter, Miami, FL) using fluorescein isothiocyanate (FITC)-labeled or phycoerythrin (PE)-labeled antibodies as previously described. Briefly, cells were collected, washed, and incubated with monoclonal mouse anti-human FITC-labeled anti-CD11b/CD11c or PE-labeled anti-CD14 (ImmunoTech, Marseille, France) for 30 minutes at room temperature. Beckton Dickinson Simultest Control (t1/r2o) was used as a negative control. Fluorescence intensity was analyzed by flow cytometry. Data were based on examination of 10 000 cells/sample selected randomly from 5 × 106 cells.

Semi-quantitative reverse transcription–PCR for PLSCR1 mRNA

Total RNA was isolated by Trizol kit (Invitrogen, Paisley, Scotland, United Kingdom) and reverse transcription (RT) was performed by TaKaRa RNA polymerase chain reaction (PCR) kit (Takara, Dalian, China) following the manufacturer’s instructions. PCR reactions to amplify PLSCR1 (first described by Sims et al21) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA were performed in a single tube using the Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with specific primers for PLSCR1 (sense strand, 5'-CAC CCT CCA TTA AAC TGT CC-3'; antisense strand, 5'-TCT TAG TGG TCT CTC CAG AG-3') and for G3PDH (sense strand, 5'-TGA AGG TCG TAC ACG TCA G-3'; antisense strand, 5'-ATG TGG GCC AGG TCC ACC AC-3'). PCR consisted of 28 cycles with denaturing at 95°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 60 seconds. Amplification cycles were preceded by a denaturation step (95°C for 5 minutes) followed by an elongation step (72°C for 10 minutes). After amplification, PCR products were analyzed on a 1% agarose gel, and the signal intensities of amplified PLSCR1 fragments were normalized against 983-bp G3PDH using a densitometer (Smartview version 5.0 software from Furi, Shanghai, China).

Quantitative real-time RT-PCR for PLSCR1 mRNA

For quantitative analysis of gene expression, total RNA was isolated by Trizol kit (Invitrogen). RNA was treated with DNase (Promega, Madison, WI). Complementary DNA was synthesized using the cDNA synthesis kit (Applied Biosystem, Foster City, CA) according to the manufacturer’s instructions. Fluorescence real-time RT-PCR was performed with the
double-stranded DNA dye SYBR Green PCR Core Reagents (PE Biosystems, Warrington, United Kingdom) using the ABI PRISM 7900 system (Perkin-Elmer, Torrance, CA). The reaction of SYBR Green assay contained 1 μL 10× SYBR Green PCR buffer, 0.8 μL deoxyxynucleoside triphosphate (dNTP) mixture, 0.1 μL AmpliTaq Gold DNA Polymerase (5 U/μL), 0.05 μL AmpErase UNG (1 U/μL), 0.05 μL forward and reverse primer (20 μM), 1 μL cDNA, and 5.65 μL double distilled H2O. The following primers were used: PLSCR1 forward, 5′-CTG ACT TCT GAG AAT GTT GC-3′ and reverse, 5′-GAA TGC TGT CGG TGG ATA CTG-3′; and β-actin forward, 5′-CAT CAC CCT GAC GTA CCC-3′ and reverse, 5′-AGC GTG AGC AAC GTA CAT G-3′. PCR was begun with one cycle of 20°C for 2 minutes (UNG inactivation) and 95°C for 10 minutes (hot-start PCR) and preceded by 40 cycles with denaturing at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. After PCR amplification cycles, a dissociation curve (melting curve) was constructed in the range of 65°C to 95°C. All amplifications and detections were carried out in a MicroAmp optical 384-well reaction plate with optical adhesive covers (Applied Biosystems, Foster, CA). PCR was done in triplicate and standard deviations representing experimental errors were calculated. All data were analyzed using ABI PRISM SDS 2.0 software (Perkin-Elmer). This software, which is coupled to the instrument, allows the determination of the threshold cycle (Ct) that represents the number of the cycle where the optical 384-well reaction plate with optical adhesive covers (Applied Biosystems, Foster, CA). PCR was done in triplicate and standard deviations representing experimental errors were calculated. All data were analyzed using ABI PRISM SDS 2.0 software (Perkin-Elmer). This software, which is coupled to the instrument, allows the determination of the threshold cycle (Ct) that represents the number of the cycle where the fluorescence intensity is significantly above the background fluorescence intensity. Using the ΔCt method, β-actin was normalized to the amount of RNA added to the reaction, and the data were subjected to cycling threshold analysis according to published procedures.

### Results

**ATRA induces PLSCR1 expression in ATRA-sensitive NB4 cells but not in ATRA-resistant NB4-derived LR1 cells**

As previously reported, ATRA at concentrations of 10^{-8} to 10^{-5} M was found to induce NB4 cells to undergo granulocytic differentiation, as assessed by morphology (data not shown) and by analysis of granulocytic differentiation–related antigens CD11b^+ and CD11c^+ /CD14^- cells (Figure 1A). Since PLSCR1 has been implicated in the maturation of granulocytes, we evaluated possible alterations of PLSCR1 expression during ATRA-induced differentiation. The results revealed that ATRA at a concentration of 10^{-6} M, which did not induce differentiation, failed to modulate the expression of PLSCR1 (Figure 1B-D). However, differentiation-inducing concentrations (10^{-8} to 10^{-5} M) of ATRA dose-dependently increased PLSCR1 protein (Figure 1B) as well as *PLSCR1* mRNA levels, as evidenced by real-time quantitative PCR (Figure 1C) and semiquantitative RT-PCR (Figure 1D). Moreover, the time course of ATRA-induced expression of *PLSCR1* (Figure 1F-G) closely paralleled that of ATRA-induced differentiation of NB4 cells (Figure 1E). Next, we treated NB4-derived maturation-resistant NB4-LR1 cells with 10^{-6} M ATRA. As shown in Figure 2A, the cell line was resistant to ATRA-induced differentiation. Furthermore, ATRA also failed to modulate PLSCR1 expression at either the mRNA (Figure 2B) or the protein level (Figure 2C) in these cells, providing further evidence for the association between ATRA-induced PLSCR1 expression and granulocytic differentiation.

### Increase in PLSCR1 expression is not limited to ATRA-induced granulocytic differentiation

To investigate whether ATRA-induced PLSCR1 expression is specific for ATRA-induced granulocytic differentiation, we treated 2 different AML cell lines, U937 and HL60, with 10^{-6} M ATRA. Under the conditions of these experiments, U937 cells differentiated to monocytes (represented by CD11b^+ /CD14^- cells, Figure 3A, left), whereas HL60 cells differentiated to granulocytes (represented by CD11b^+ /CD14^-, Figure 3A, right). PLSCR1 expression was found to be significantly elevated by ATRA, irrespective of whether ATRA induced granulocytic (HL60) or monocytic (U937) differentiation (Figure 3B-C).

Next, we investigated the effects of other known differentiation-inducing agents, including SB (2 mM), DMSO (1%), PMA
(100 nM), and VD3 (2.5 × 10⁻⁷ M), on PLSCR1 expression in NB4 and U937 cells (Figure 4A-B). Although SB, a histone deacetylase inhibitor, and DMSO could effectively induce NB4 and U937 cells to undergo granulocytic differentiation, they only slightly up-regulated PLSCR1 mRNA and protein. VD3, which induced monocytic differentiation, did not alter PLSCR1 expression in these cells. By contrast, PMA, which induced monocytic differentiation, significantly enhanced PLSCR1 expression comparable with ATRA. Of note, 1% DMSO and 100 nM PMA also increased PLSCR1 expression in HL60 cells (Figure 4C and data not shown). These results suggested that whereas an increase in PLSCR1 expression is observed in ATRA- or PMA-induced cell differentiation, increased PLSCR1 expression might not be necessary when differentiation is induced by other factors in NB4 and U937 cells.

Role of PKC in ATRA/PMA-induced PLSCR1 expression

The increase in PLSCR1 expression induced by ATRA and PMA was not restricted to leukemic cells. As shown in Figure 4D, both ATRA at 10⁻⁶ M and PMA at 2 to 5 × 10⁻⁷ M also increased expression of PLSCR1 in Cos-7 cells. This suggested that ATRA and PMA might regulate PLSCR1 expression via a common mechanism that is independent of the cell type. As depicted in Figure 5A, both PMA and ATRA, but not DMSO and VD3, activated PKC, as evidenced by phosphorylation of PKC on Serine 643 in NB4 cells (Figure 5A) and U937 cells (data not shown). These results suggested that whereas an increase in PLSCR1 expression is observed in ATRA- or PMA-induced cell differentiation, increased PLSCR1 expression might not be necessary when differentiation is induced by other factors in NB4 and U937 cells.

Role of PKCα in ATRA/PMA-induced PLSCR1 expression

The increase in PLSCR1 expression induced by ATRA and PMA was not restricted to leukemic cells. As shown in Figure 4D, both ATRA at 10⁻⁶ M and PMA at 2 to 5 × 10⁻⁷ M also increased expression of PLSCR1 in Cos-7 cells. This suggested that ATRA and PMA might regulate PLSCR1 expression via a common mechanism that is independent of the cell type. As depicted in Figure 5A, both PMA and ATRA, but not DMSO and VD3, activated PKCα, as evidenced by phosphorylation of PKCα on Serine 643 in NB4 cells (Figure 5A) and U937 cells (data not shown).
PKCδ regulates PLSCR1 expression

PLSCR1 is not phosphorylated by PKCδ

The observation that PKCδ could up-regulate PLSCR1 (Figure 5C) raised the question of whether PLSCR1 was being phosphorylated by PKCδ, as has been reported for in vitro phosphorylation of PLSCR1 immunoprecipitated from cells, and for in vivo phosphorylation in apoptotic Jurkat cells. For these experiments, PLSCR1 purified from human erythrocytes was used as substrate in an in vitro phosphorylation assay with recombinant PKCδ. As shown in Figure 6A, phosphorylation of PLSCR1 was not observed under these conditions, even though autophosphorylation of PKCδ and phosphorylation of histone were readily detected. Intracellular phosphorylation of PLSCR1 was also not observed when apoptosis was induced in Jurkat cells by Fas ligation (Figure 6B), in contrast to the report by Frasch et al. Taken together, our results suggest that PLSCR1 is not a substrate for phosphorylation by PKCδ.

Inhibition of PLSCR1 expression by siRNA partially blocks ATRA- and PMA-induced differentiation

We investigated a possible role for PLSCR1 in leukemic cell differentiation by blocking PLSCR1 expression with siRNA. Of the 5 target sequences we selected to silence PLSCR1 expression, stable transfection of U937 cells with P2 and P5 reduced basal, and significantly inhibited ATRA- and PMA-induced PLSCR1 expression (Figure 7A and data not shown). Of note, P5 siRNA was more effective in suppressing PLSCR1 expression than P2 siRNA. Interestingly, stable transfection with P2 and particularly P5 siRNA also significantly inhibited ATRA- and PMA-induced cell differentiation, as evidenced by CD11b expression (Figure 7B) and morphologic features (Figure 7C), while differentiation induced by DMSO, VD3, and SB was not affected (data not shown), strongly indicating a role for PLSCR1 in ATRA/PMA-induced leukemic cell differentiation.
leukemic cell differentiation has attracted significant attention. Because PLSCR1 has previously been implicated in the proliferation and terminal differentiation of myeloid precursor cells, and a truncated mutation of PLSCR1 has been reported to confer a leukemogenic phenotype, we sought to investigate the role of differentiation-inducing agents on cellular PLSCR1 expression. Nakamaki et al reported the specific induction of PLSCR1 mRNA upon granulocytic differentiation of the promyelocytic leukemia NB4 and HT93 cells by ATRA. By contrast, no increase in PLSCR1 mRNA was observed when the bipotential myeloid leukemia HL-60 cells were induced to differentiate toward monocytes/macrophages, during erythroid differentiation induced by hemin in erythroid leukemia K562 and HEL cells or during megakaryocytic differentiation induced by PMA in K562 cells. In the present study, we showed that pharmacologic concentrations of ATRA elevated PLSCR1 mRNA and protein levels in NB4 and HL-60 cells with the induction of differentiation toward granulocytes. However, by contrast to the report of Nakamaki et al., PLSCR1 was also up-regulated upon ATRA-induced monocytic differentiation of U937 cells. Additionally, PMA, which induced these cells (NB4, U937 and HL60) to differentiate toward the monocytic phenotype, also potently enhanced PLSCR1 expression. Moreover, only minimal or no elevation of PLSCR1 was observed upon treatment with other granulocytic or monocytic differentiation-inducing agents, including DMSO, SB, or VD3 in NB4 and U937 cells, although elevated expression of PLSCR1 was seen in DMSO-treated HL60 cells. These results indicated that a downstream signaling pathway common to both ATRA and PMA might contribute to the regulation of PLSCR1 expression, which was further supported by the fact that PMA and ATRA also up-regulated PLSCR1 expression in Cos-7 cells. Since PMA is a strong activator of many isoforms of PKC, and ATRA has been shown to directly bind to PKC isoforms and modulate the activity of PKCδ, we hypothesized that PKCδ was mediating the response of PLSCR1 expression to these 2 differentiation-inducing agents. Indeed, the PKCδ-specific inhibitor rottlerin almost completely abrogated ATRA- and PMA-induced PLSCR1 expression, providing the first evidence for a role of PKCδ in PLSCR1 expression. The mechanism by which PKCδ up-regulates PLSCR1 expression remains to be investigated. We speculate that it is likely indirect, and not through phosphorylation of PLSCR1. Although PKCδ has been previously reported to phosphorylate PLSCR1 with consequent activation of PL scramblase activity when coexpressed in...
Chinese hamster ovary (CHO) cells, or in apoptotic cells, they have been unable to demonstrate PKCδ-mediated phosphorylation of purified PLSCR1 directly in vitro, and by contrast to the report by Frasch et al., we did not observe phosphorylation of PLSCR1 in apoptotic Jurkat cells. The discrepancy remains unresolved.

In a recent report, Kambhampati et al. demonstrated activation of PKCδ upon ATRA-induced differentiation of NB4 and HL-60 cells, and inhibition of PKCδ activity abrogated ATRA-induced cell differentiation, suggesting a critical role for PKCδ in mediating the biologic effects of ATRA in malignant cells. Furthermore, they showed that PKCδ forms a complex with the retinoic acid receptor α (RARα) and binds to retinoic acid–responsive elements (RAREs), and that inhibition of PKCδ blocked ATRA-dependent gene transcription via RARE. Exactly how activation of PKCδ mediates leukemic cell differentiation is unknown. Here we demonstrate that in maturation-resistant, ATRA-responsive NB4-LR1 cells, up-regulation of PLSCR1 by ATRA is not observed, suggesting a possible role for PLSCR1 in ATRA-induced cell differentiation. Consistent with this, silencing of PLSCR1 expression with siRNA inhibits ATRA- and PMA-induced cell differentiation, as assessed by morphologic (Figure 7C) and functional (increased CD11b expression, Figure 7B) criteria. Of note, antisense PLSCR1 transfection was also shown to significantly suppress ATRA-induced differentiation of NB4 cells. Taken together, these results indicate that as a protein that is induced upon PKCδ activation, PLSCR1 is required for leukemic cell differentiation by these agents. Whether PLSCR1 affects cell differentiation through its activity at the plasma membrane or whether ATRA- and PMA-induced PLSCR1 exerts its effects following its translocation into the nucleus remains to be elucidated.

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