Phospholipid Scramblase 1 Binds to the Promoter Region of the Inositol 1,4,5-Triphosphate Receptor Type 1 Gene to Enhance Its Expression*

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Phospholipid scramblase 1 (PLSCR1) is a multiply palmitoylated, endofacial membrane protein originally identified based on its capacity to promote accelerated transbilayer phospholipid movement in response to Ca\(^{2+}\). Recent evidence suggests that this protein also participates in cell response to various growth factors and cytokines, influencing myocardial differentiation, tumor growth, and the antiviral activity of interferon. Whereas plasma membrane PLSCR1 was shown to be required for normal recruitment and activation of Src kinase by stimulated cell surface growth factor receptors, PLSCR1 was also found to traffic into the nucleus and to tightly bind to genomic DNA, suggesting a possible additional nuclear function. We now report evidence that PLSCR1 directly binds to the promoter region of the inositol 1,4,5-triphosphate receptor (IP3R1) to enhance expression of the receptor. Probing a mapped to residues 101-GTAACCATGTGGA-89, and the segment spanning Gln\(^{106}\)-Glu\(^{118}\) in PLSCR1 was identified to mediate its transcriptional activity. The significance of this interaction between PLSCR1 and IP3R1 in situ was confirmed by comparing levels of IP3R1 mRNA and protein in matched cells that either expressed or were deficient in PLSCR1. These data suggest that in addition to its role at the plasma membrane, effects of PLSCR1 on cell proliferative and maturation responses may also relate to alterations in expression of cellular IP3 receptors.

The phospholipid scramblase (PLSCR) family gene consists of an apparent tetrad of genes with identifiable orthologues conserved from Caenorhabditis elegans to man (1). The first identified member of this family (PLSCR1) was isolated based upon its capacity to promote Ca\(^{2+}\)-dependent accelerated transbilayer membrane phospholipid (PL) movement, mimicking the remodeling of plasma membrane PL that is observed under conditions of injury and apoptosis (2, 3). PLSCR1 is a multiply palmitoylated, Ca\(^{2+}\)-binding, Pro- and Cys-rich, endofacial plasma membrane protein that was shown to distribute into lipid raft domains and to be a substrate of the Abl and Src tyrosine kinases (4–6). The exact biologic function of this protein remains controversial; although PLSCR1 mediates Ca\(^{2+}\)-dependent transbilayer movement of PL in proteoliposomes (2, 3) and was reported to increase cell surface expression of phosphatidyserine through remodeling of plasma membrane PL in mammalian cells exposed to Ca\(^{2+}\) ionophore and other inducers of injury or apoptosis (7–9), it has also been observed that induction of PLSCR1 expression can occur without a detectable increase in PL movement between membrane leaflets, and gene deletion of PLSCR1 did not impair cell capacity to undergo this remodeling of cell surface PL (10–12). Whereas its role in plasma membrane PL movement remains unresolved, there is increasing evidence that PLSCR1 serves to regulate cellular maturation and proliferative responses; mutation in PLSCR1 was shown to influence the leukemic potential of the murine monocyte cell line MM, whereas gene deletion of PLSCR1 or inhibition of PLSCR1 protein expression using antisense or small interfering RNA was found to impair myeloid cell maturation or proliferation in response to select hematopoietic growth factors (12–16). PLSCR1, a potentially stimulated interferon (IFN)-responsive gene, was also found to be required for normal expression of the antiviral activity of IFN, whereas PLSCR1 gene deletion and small interfering RNA suppression of PLSCR1 expression were found to inhibit the expression of a select subset of IFN-stimulated genes, including those with known antiviral activity (17, 18). The mechanism by which PLSCR1 influences cell response to these various growth factors and cytokines remains unresolved. In the case of epidermal growth factor receptor, PLSCR1 was shown to be recruited into the activated plasma membrane epidermal growth factor receptor complex, concomitant with the phosphorylation of PLSCR1 by activated Src and its binding to the phosphotyrosine domain of the adapter Shc (4, 5). Gene deletion of PLSCR1, or mutation of its Tyr residues that are phosphorylated by Src, prevented this interaction and was accompanied by attenuated growth factor-induced activation of Src kinase, implying a role for PLSCR1 in Src recruitment and feedback activation of the kinase by activated epidermal growth factor receptor. Similar interactions were also observed with related growth factor receptors.

Despite the considerable evidence that PLSCR1 is an endofacial cell surface protein with apparent biologic function at the plasma membrane, recent data suggest an additional role for this protein in the nucleus. Nuclear trafficking of PLSCR1 has been observed only in circumstances where its cellular expression was induced by IFN and other cytokines or growth factors that transcriptionally activate this gene, implying nuclear import of de novo synthesized PLSCR1 rather than a redistribution of the membrane-bound pool (19). Mutation of PLSCR1 at its sites of Cys palmitoylation or inhibition of palmitoylation by cell treatment with bromo-palmitate also resulted in nuclear trafficking of...
the protein and abrogated its distribution to the cell surface. Nuclear import of PLSCR1 was shown to be mediated by the importin nucleopor transport chaperones and to be dependent on specific binding of PLSCR1 to importin-α via an unconventional nuclear localization signal identified within the PLSCR1 polypeptide (20). PLSCR1 was also found to bind directly to genomic DNA, and after nuclear uptake, its extraction from the nucleus required salt elution or incubation with DNase, implying interaction with chromatin.

To gain insight into the possible function of nuclear PLSCR1, we utilized genomic binding site cloning (GBSC) from a human CpG island-enriched library to identify potential gene regulatory regions to be utilized. Genomic binding site cloning (GBSC) from a human CpG island genomic library in E. coli (UK HGMP Resource Center) was amplified in 0.3% low melting agarose-LB medium, and the DNA was purified with a Qiagen column. Genomic DNA inserts (average size, 760 bp) were amplified by PCR for 30 cycles using the primers 5′-CGG CCG CCT GCA GGT CGA CCT TAA-3′ and 5′-AAC GCG TTG GGA GCT CTC CTT CCT ATT-3′ according to the manufacturer’s instructions. Amplified genomic DNA was purified by phenol extraction.

Preparation of CpG Island Genomic Library DNA—Human CpG island genomic library in E. coli (UK HGMP Resource Center) was amplified in 0.3% low melting agarose-LB medium, and the DNA was purified with a Qiagen column. Genomic DNA inserts (average size, 760 bp) were amplified by PCR for 30 cycles using the primers 5′-CGG CCG CCT GCA GGT CGA CCT TAA-3′ and 5′-AAC GCG TTG GGA GCT CTC CTT CCT ATT-3′ according to the manufacturer’s instructions. Amplified genomic DNA was purified by phenol extraction.

Genomic DNA-binding Site Cloning—PCR-amplified CpG library genomic DNA was precloned by incubation of 25 μg of DNA with 250 μl of GST immobilized on glutathione-Sepharose 4B (GST-Sepharose) and 400 μl of binding buffer (40 mM Tris, pH 7.5, 100 mM KCl, 10% glycerol, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1% Triton X-100, and protease inhibitor mixture) for 1 h at 4°C. The precloned genomic DNA was incubated with 21 μl (10 μg of protein) of either PLSCR1-GST immobilized on glutathione-Sepharose 4B (PLSCR1-GST-Sepharose) or control GST-Sepharose for 1 h at 4°C with agitation. The beads were washed five times with binding buffer and suspended in 300 μl of PCR buffer (BD Clontech). Bound genomic DNA was amplified by PCR for 25 cycles (denaturing for 1 min at 94°C, annealing for 0.5 min, and extension for 1.5 min at 68°C) with Advantage DNA polymerase and the primers noted above. PCR products were purified by phenol extraction and used in the next cycle of GBSC. After seven cycles of GBSC, enriched genomic DNA was separated on agarose gel and cloned into Topo-pCR2.1 T-A cloning vector (Invitrogen), and the inserts were sequenced.

Electrophoretic Mobility Shift Assay (EMSA)—Genomic DNA fragments from GBSC were end-labeled with [γ-32P]ATP (Amersham Biosciences) by T4 polynucleotide kinase (New England Biolabs). Three μl of labelled DNA (3 x 10^6 cpm) were mixed with 19 μl of EMSA binding buffer (10 mM HEPES, pH 7.7, 50 mM KCl, 1 mM MgCl2, 1 mg/ml bovine serum albumin, 10% glycerol, 0.5 mM dithiothreitol, 100 μg/ml poly(dI-dC)) and 3 μl of either human PLSCR1-MBP (1.2 μg) or MBP (1.2 μg). After incubation for 45 min at 4°C, PLSCR1-complexed 32P-DNA probe was separated from free 32P-DNA probe on a 6% DNA retardation gel (Invitrogen) and detected by exposure to x-ray film (Kodak).

Mapping Gene Transcriptional Activation Domain in PLSCR1—Full-length PLSCR1 cDNA (Met1-Trp318) or its serial deletions (Met1-Trp318, Val11-Trp318, Arg7-Trp318, and Pro21-Trp318) were cloned into pAS2–1 vector (BD Clontech) to encode fusion proteins with Gal4 DNA-binding domain. Yeast was transformed with either full-length PLSCR1-pAS2–1 or the PLSCR1 deletions or pAS2–1 vector control, and cultured on agar plates. Yeast colonies were transferred to filter paper, and expression of β-galactosidase under the control of Gal4-responsive elements was detected with X-GAL as substrate. Colonies were imaged using Kodak Image Station 440 CF (PerkinElmer Life Sciences).

PLSCR1- and PLSCR3-deficient Mice and Cell Lines—PLSCR1-/- and PLSCR3-/- mice were produced by Lexicon Genetics Incorporated (The Woodlands, TX) as previously described (12, 35). Mice deficient in both PLSCR1 and PLSCR3 (PLSCR1(1&3)-/-) were produced by breeding of PLSCR1-/- with PLSCR3-/- mice. Embryonic fibroblasts (MEF) and kidney epithelial cells (KEC) from PLSCR1-/- and PLSCR1(1&3)-/- mice were immortalized with SV40 and cloned as previously described (4). Clonal cell lines were then cultured with the murine stem cell virus containing either MCV-ires-GFP (vector control) or the following MSCV-PLSCR1-ires-GFP constructs: wild type PLSCR1; PLSCR1 184CCCPCC189 → 184AAAAA189 (PLSCR1-C→A), a nuclear trafficking PLSCR1 construct in which plasma membrane attachment is prevented by mutation of all palmitoylated residues; the PLSCR1 double mutant control 184CCCPCC189 → 184AAAAA189, 257GKISKHWTGPG184 → 257GAISAAWWTGPG (PLSCR1-C→A&K→A), in which plasma membrane attachment as well as nuclear trafficking are prevented by mutation of both the palmitoylated residues and the nuclear localization signal in PLSCR1 (19, 20). Prior to use, the cells were sorted by flow cytometry to achieve comparable GFP expression.

Cell Culture and Treatment—Sorted MEF were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and KEC in Dulbecco’s modified Eagle’s medium and F-12 (1:1), 10% fetal bovine serum. In some cases, the cells were treated with or without 2 μM all-trans-retinoic acid (ATRA) (Sigma) for 24 h and harvested to measure gene expression by Northern and Western blots as described below.

Transcriptional Activity Assay—PLSCR1 or PLSCR1-C→A (184CCCPCC189 → 184AAAAA189) mutant were cloned into pcDNA3, and GBSC-derived IP3R1 genomic DNA was cloned into pGL3 (Promega). Immortalized PLSCR1(1&3)-/- MEF were co-transfected with 1.4 μg of pcDNA3-PLSCR1 or pcDNA3-PLSCR1-C→A (184CCCPCC189 → 184AAAAA189) mutant, 0.1 μg of pGL3-GBSC-derived genomic DNA, and 0.1 μg of PRL-TK (Promega) for calibration of transfection efficiency, using the Lipofectamine plus reagent (Invitrogen). Forty-eight hours after transfection, luciferase activity was assayed with the dual luciferase system (Promega). Relative luciferase activity is presented as a ratio of firefly luciferase to Renilla luciferase activity (Promega). The results are shown as ratios of luciferase to GFP values.

Northern Blot—Total RNA in MEF was purified using the RNeasy kit (Qiagen). Eight μg of total RNA in 30 μl RNA sample buffer were loaded onto a 1% formaldehyde denaturing agarose gel. After electrophoresis the RNA was transferred to a positively charged nylon membrane.
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FIGURE 1. PLSCR1 binds to GBSC-derived genomic DNA. Four genomic DNA clones (1, 2, 3, and 4) were isolated from a human Cpg island genomic DNA library by GBSC, labeled with $^{32}$P, and incubated with either PLSCR1-MBP or control MBP. PLSCR1/32P-DNA complexes (indicated by arrow) were separated from free $^{32}$P-DNA probes (bottom) on a 6% DNA retardation gel. The data of a single experiment are shown, representative of three so performed. GBSC3 contains 191 bp within the promoter region and exon 1 of IP3R1. The sequences of GBSC1 (235 bp), GBSC2 (242 bp), and GBSC4 (152 bp) correspond to the uncharacterized human genomic sequence.

Western Blot—Mouse primary bone marrow cells, MEF, and KEC were incubated for 1 h at 4 °C in cell lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture) and centrifuged at 20,800 g for 10 min at 4 °C. Brain tissue was isolated from either mice and rats or incubated with either the Gal4 DNA-binding domain or an intrinsic regulatory factor in the reporter cell with such an activation domain. Expression of PLSCR1 was expressed as a fusion protein with the Gal4 DNA-binding domain, and Pro177–Trp318) were fused with Gal4 DNA-binding domain and expressed as fusion proteins in yeast. $\beta$-Galactosidase was measured as described under “Experimental Procedures.” Colonies indicate PLSCR1-activated $\beta$-galactosidase gene transcription (A). The results suggest that the putative transcriptional activation domain in PLSCR1 maps to residues Met86–Glu118 (B).

RESULTS

Identification of PLSCR1-binding DNA—Because PLSCR1 was shown to be actively imported into the nucleus and to avidly bind genomic DNA, we sought to identify candidate DNA sequences that bound PLSCR1 within a Cpg island-enriched human genomic library. Seven rounds of panning and amplification against this library using GST-PLSCR1 as bait yielded four distinct clones that bind to PLSCR1 (Fig. 1): three with inserts that corresponded to sequence derived from uncharacterized genes and one with a sequence from the 5′ regulatory region of IP3R1 (residues –112 to +79, of Ensembl sequence ENST00000302640 at www.ensembl.org). The specificity of the clones that were isolated on GST-PLSCR1 for binding to PLSCR1 was confirmed by performing EMSA against MBP-PLSCR1. As shown in Fig. 1, a shift in mobility was observed for all four clones in the presence of MBP-PLSCR1 but not in the presence of MBP alone.

Identification of a Gene Transcriptional Activation Domain in PLSCR1—Next we used a yeast Gal4-LacZ reporter system to examine whether PLSCR1 contains a transcriptional activation domain. When PLSCR1 was expressed as a fusion protein with the Gal4 DNA-binding domain, transcriptional activity as detected by $\beta$-galactosidase gene expression was observed, in absence of a “bait” library containing the requisite transcription activation domain for the LacZ reporter. As expected, expression of the Gal4 DNA-binding domain alone did not activate transcription (Fig. 2). This suggested that PLSCR1 directly activated the minimal promoter of LacZ or interacted with an intrinsic factor in the reporter cell with such an activation domain. Expression of a series of truncated mutants of PLSCR1 revealed that PLSCR1-dependent transcriptional activity was lost upon truncation between residues Met86 and Glu118, locating a putative transcriptional activation domain to this 33-amino acid segment within PLSCR1.

PLSCR1 Promotes IP3R1 Gene Transcription—Having demonstrated specific binding of PLSCR1 to DNA sequences identified by GBSC and having located a putative transcription activation domain within PLSCR1, we next investigated the effect of PLSCR1 on gene transcription in a mammalian cell line. For these experiments, the DNA inserts of the four GBSC-derived clones were cloned into the luciferase reporter vector pGL3. Because the PLSCR1 protein sequence is highly homologous to PLSCR3 and shows similar tissue distribution, a cell line deficient in both PLSCR1 and PLSCR3 (PLSCR1&3)−/− MEF was
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A mutant PLSCR1 without potential interference by PLSCR3. As shown in Fig. 3, GBSC clone 3 (representing nucleotides 184 AAAPAA 189), PRL-TK vector, and with either pGL3-GBSC-derived genomic clones 1–4 or pGL3 vector control. Forty-eight hours post-transfection, luciferase activity was measured with correction for transfection efficiency. Closed and open bars represent cells transfected with PLSCR1 C→A mutant or vector only, respectively. The data of a single experiment are shown, representative of five so performed. B, PLSCR(1&3)/→/MEF were transiently co-transfected with pEGFP-C3 vector, either wild type PLSCR1 or PLSCR1 C→A mutant, and either pGL3-IP3R1 promoter (→112 to +79) or pGL3 vector. Luciferase activity was measured with correction for transcription efficiency. Closed and open bars represent transcriptional activities measured for cells in the presence and absence of IP3R1 promoter, respectively. The data of a single experiment are shown, representative of four so performed. See “Experimental Procedures” for details.

selected for these experiments to analyze the transcriptional activity of PLSCR1 without potential interference by PLSCR3. As shown in Fig. 3A, of the four GBSC-derived clones that bound PLSCR1 (from Fig. 1), only GBSC clone 3 (representing nucleotides →112 to +79 of the IP3R1 promoter region) was found to activate luciferase gene expression of the pGL3 reporter when co-transfected with PLSCR1. This activation of the IP3R1 (→112 to +79)-pGL3 reporter construct by PLSCR1 was observed for both the wild type protein and for the PLSCR1-C→A mutant, which localizes preferentially to the nucleus (Fig. 3B).

To identify the sequence in the IP3R1 promoter region required for the observed PLSCR1-mediated gene transcription, pGL3 luciferase reporter constructs containing the full-length or serial deletions of the IP3R1 promoter region (→112 to +79) were transfected into PLSCR1(1&3)/→/MEF for evaluation of gene transcription activity. As shown in Fig. 4, truncation of 33 bp from the 5′ end (construct →79 to +79) resulted in the loss of >90% of transcriptional activity, suggesting that the sequence from →112 to +80 of the IP3R1 promoter region was primarily responsible for the observed PLSCR1-enhanced reporter gene transcription. However, a pGL3 luciferase reporter construct containing nucleotides →112 to +80 exhibited <10% of the transcriptional activity of the full-length construct, indicating an additional contribution by sequence contained within nucleotides →79 to +79. To identify the binding site for PLSCR1 within the IP3R1 promoter region, full-length and the partial IP3R1 promoter sequences were subjected to EMSA with PLSCR1. Oyly full-length (→112 to +79) and the sequence from →112 to +80 were observed to bind to PLSCR1, locating the binding site to this 33-bp sequence (not shown). The binding site for PLSCR1 within this sequence was further delineated by performing EMSA with PLSCR1 of serial mutations of this 33-bp segment (Fig. 5). These experiments mapped the minimal binding site for PLSCR1 to 13 nucleotides (→103 GTTACCATGTGGA −89) of the IP3R1 promoter region. This 13-bp sequence containing the PLSCR1-binding site in human IP3R1 is identical conserved in mouse IP3R1.

Finally, we examined the effect of PLSCR1 on cellular IP3R1 gene expression in situ. IP3R1 is constitutively expressed in many mamma-
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FIGURE 6. Ectopic expression of PLSCR1 enhances IP3R1 gene transcription. PLSCR1(+3)−/− MEF were stably transfected with MSCV-PLSCR1-C→A mutant-IRES-GFP, MSCV-PLSCR1 (WT)-IRES-GFP, or MSCV-IRES-GFP, respectively. The cells were incubated with or without 2 μM ATRA for 24 h. Total RNA was purified, and cell lysates were prepared. A, Northern blot of IP3R1, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The normalized band intensities were as follows: IP3R1, −ATRA, PLSCR1-C→A, 3.9, and PLSCR1(WT), 2.4; vector, 1.0, IP3R1, +ATRA, PLSCR1-C→A, 2.6, and PLSCR1(WT), 1.5; vector, 1.0. B, Western blot of indicated antigens, with β-actin as loading control. The normalized band intensities were as follows: IP3R1, −ATRA, PLSCR1-C→A, 2.9, and PLSCR1(WT), 0.9; vector, 1.0, IP3R1, +ATRA, PLSCR1-C→A, 2.2, and PLSCR1(WT), 0.8; vector, 1.0, IP3R1, −ATRA, PLSCR1(C→A), 0.8, and PLSCR1(WT), 0.6; vector, 1.0, IP3R2, +ATRA, PLSCR1-C→A, 0.7, and PLSCR1(WT), 0.8; vector, 1.0, IP3R3, −ATRA, PLSCR1-C→A, 1.5, and PLSCR1(WT), 1.5; vector, 1.0, IP3R3, +ATRA, PLSCR1-C→A, 1.0, and PLSCR1(WT), 1.0; vector, 1.0. The data of a single experiment are shown, representative of three so performed.

FIGURE 7. PLSCR1 increases IP3R1 expression in mammalian cells. Cell lysates of mouse brain, bone marrow cells (BMC), and SV40 immortalized KEC of the indicated genotype were prepared, and IP3R1, PLSCR1, and β-actin were detected by Western blot as described under “Experimental Procedures” (A and B). In C, PLSCR1(+3)−/− KEC were stably transfected with either MSCV-PLSCR1-C→A mutant-IRES-GFP, or MSCV-PLSCR1(C→A) mutant-IRES-GFP, or MSCV-PLSCR1 (WT)-IRES-GFP, or MSCV-PLSCR1(C→A) mutant-IRES-GFP, or MSCV-IRES-GFP vector control. The cells were incubated with 2 μM ATRA for 24 h, the cell lysates were prepared, and IP3R1, PLSCR1, and β-actin were detected by Western blot. The normalized band intensities were as follows: A, Brain-IP3R1, WT, 1.7, and PLSCR1−/−, 1.0; BMC-IP3R1, WT, 3.6, and PLSCR1−/−, 1.0; 8. B, MEF-IP3R1, WT, 1.7, and PLSCR1−/−, 1.0; BMC-IP3R1, WT, 3.6, and PLSCR1−/−, 1.0; BMC-IP3R1, −ATRA, 1.9, and PLSCR1−/−, 1.2, and PLSCR1-C→A & K→A, 1.0; KEC-IP3R1, WT, 1.7, and PLSCR1−/−, 1.0; KEC-IP3R1, −ATRA, 1.9, and PLSCR1−/−, 1.2, and PLSCR1-C→A & K→A, 1.0; vector, 1.0; IP3R1, −ATRA, PLSCR1-C→A, 2.2, and PLSCR1(WT), 0.9, and PLSCR1-C→A & K→A, 1.0; vector, 1.0. Decreased protein expression of PLSCR1-C→A & K→A construct may reflect accelerated degradation of this double mutant control in the cytosol (see text). The data of a single experiment are shown, representative of two so performed.

PLSCR1, its expression has been shown to increase in cells treated with retinoic acid. In these experiments, PLSCR1(+3)−/− MEF stably transfected with wild type, mutant PLSCR1-C→A, or vector control were subjected to Northern blotting for IP3R1 mRNA and Western blotting for IP3R1 protein. As shown in Fig. 6, when compared with the vector only controls, cells expressing the nuclear localized PLSCR1 mutant (PLSCR1-C→A) exhibited >2-fold increase in expression of IP3R1 mRNA (Fig. 6A) and protein (Fig. 6B) under both basal and ATRA-stimulated conditions. Ectopic expression of wild type PLSCR1, which is normally palmitoylated and predominantly distributes to plasma membrane and not to nucleus (19), resulted in only small and variable changes in either basal or ATRA-stimulated expression of IP3R1. By contrast to the observed elevation in IP3R1 expression in cells expressing the nuclear trafficking PLSCR1 mutant, we detected no consistent change in the levels of expression of either IP3R2 or IP3R3 (Fig. 6B). Consistent with these data, inspection of the promoter segments of the IP3R2 and IP3R3 genes also failed to reveal nucleotide sequence corresponding to the deduced PLSCR1-binding site in IP3R1 (GTAAC-CATGTTGGA; see Fig. 5).

In addition, an effect of PLSCR1 on constitutive IP3R1 expression was also observed when primary cells and tissue harvested from WT mice versus PLSCR1−/− mice were compared. As shown by Western blots of Fig. 7A, whole cell extracts of brain and bone marrow from PLSCR1−/− mice consistently exhibited lower levels of IP3R1 protein expression than corresponding cells obtained from wild type animals. Similarly, diminished IP3R1 expression was also observed in immortalized kidney epithelial cells obtained from PLSCR1(+3)−/− mice compared with identical cells from wild type animals (Fig. 7B).

As was observed for PLSCR1(+3)−/− MEF (Fig. 6), the expression of IP3R1 in PLSCR1(+3)−/− KEC was found to increase upon ectopic expression of PLSCR1 in the cell, and this activity depended upon the capacity of PLSCR1 to enter the nucleus; reconstitution of these cells with the palmitoylation-defective PLSCR1-C→A mutant, which localizes to the nucleus, increased cellular IP3R1 levels under both basal and ATRA-stimulated conditions (Fig. 7C). By contrast to this nuclear-trafficking PLSCR1 mutant, cells transfected to express wild type PLSCR1, which distributes to the plasma membrane and not to the nucleus, showed no increase in IP3R1 expression relative to matched vector controls. We also observed no increase in IP3R1 expression from vector
levels in PLSR1(-3)/- KEK transfected with the double mutant PLSR1-C→A&K→A, a palmitoylation-defective mutant with additional mutation in the PLSR1 nuclear localization signal to prevent its nuclear entry. Of note, however, whereas the PLSR1(-3)/- KEK used in these experiments were matched in both GFP fluorescence and in expressed mRNA (data not shown), the level of expression of the PLSR1-C→A&K→A double mutant was always distinctly less than that of the WT or PLSR1-C→A constructs (Fig. 7C), potentially reflecting accelerated degradation of the double mutant construct in the cytosol.

**DISCUSSION**

These experiments identify a gene that is directly transcriptionally regulated by PLSR1, a multiply palmitoylated plasma membrane protein that was recently discovered to traffic into the nucleus as cargo of the importin nucleoporop protein and to bind directly to DNA. The gene target of nuclear PLSR1, IP3R1, is known to play a key role in IP3-mediated mobilization of intracellular Ca2+ stores from the endoplasmic reticulum of a variety of cells and tissues. This transcriptional activity associated with PLSR1 toward the cellular IP3R1 gene was found to reflect 1) specific binding of PLSR1 to the nucleotide sequence -101 GTACAAGGTGGA 89 that is conserved within the promoters of both human and mouse IP3R1 (Fig. 5); 2) an apparent transcription activation domain within the PLSR1 peptide segment Met86–Glu118 (Fig. 2); and 3) the capacity of PLSR1 for nuclear import via a nuclear localization signal previously identified within PLSR1 peptide segment 257 GKISKHTGI266 (Fig. 7C). Unresolved by these experiments is whether PLSR1 functions in situ as a direct transcription factor to drive IP3R1 expression or as a co-activator to enhance the transcriptional activity of another transcription factor acting at the IP3R1 promoter.

The PLSR1-binding site identified in IP3R1 (−101 to −89) is close to both a "TATA box" (−131 to −126) and to the binding site of transcription factor AP-2 (−191 to −184), which has been shown to regulate IP3R1 gene transcription (21). This suggests that once bound to the promoter, PLSR1 might either directly promote transcription of IP3R1 through its own transcriptional activation domain (Fig. 2) or enhance the transcriptional activity of AP-2 or other transcription factor acting on the gene. Also of note, this binding site for PLSR1 within the IP3R1 promoter (−101 GTACAAGGTGGA 89, Fig. 5) itself contains an "E box" core consensus sequence, which has been identified as the target of many basic helix-loop-helix transcription factors and co-activators (22–25). It remains to be determined whether nuclear PLSR1 also binds to other genes that contain this sequence so as to influence their transcription.

IP3 receptors (types 1, 2, and 3) are intracellular Ca2+ release channels that respond to the second messenger IP3 upon activation of a variety of cell surface receptors. By regulating the intracellular release of endoplasmic reticulum calcium stores, the IP3 receptors play central roles in regulating diverse cell responses to external stimuli, including storage, secretion, exocytosis/contraction, gene expression, cell growth, differentiation, and maturation (26–30). Among the three IP3 receptors, IP3R1 is widely distributed in a variety of cells and tissues, including most notably, prominent expression in Purkinje and other neuronal cells of the central nervous system, smooth muscle, cardiomyocytes, various epithelial cells, and oocytes (28, 29, 31). In many cells, IP3R1 expression is up-regulated in response to ATRA, 1,25-dihydroxyvitamin D3, and interleukin-1B, and direct transcriptional regulation of the IP3R1 promoter has been demonstrated for transcription factors AP-2 and neuro-D-related factor (21, 32–34). In this context, it is of particular interest to note 1) recent evidence that cellular expression of PLSCR1 is also up-regulated by ATRA and that this induced expression of PLSCR1 in myeloid cell lines has been shown to be required for normal differentiation and maturation in response to ATRA (12–14) and 2) the substantial reduction in IP3R1 expression that was observed in cells deficient in PLSCR1 (Figs. 6 and 7). Of interest, available evidence suggests that the IP3R1 promoter lacks functional retinoic acid response elements, which implies that its activation in cells treated with ATRA is mediated by other factor(s) that are activated or transcriptionally induced by ATRA. Because the expression of PLSCR1 is induced by ATRA, and this protein exhibits transcriptional activity at the IP3R1 promoter, its participation in feedback activation of IP3R1 expression through ATRA is suggested. A similar role has been proposed for AP-2, an ATRA-induced transcription factor that binds to its site in the IP3R1 promoter to enhance transcription of the gene (21). Alternatively, the binding of PLSCR1 to its site in the IP3R1 promoter (−101 GTACAAGGTGGA 89), which is in the vicinity of the AP-2-binding site (−191 to −184), might function to enhance AP-2-mediated transcriptional activation.

In addition to its potential role in regulating cellular content of IP3R1, it is of interest to note that the expression of PLSCR1, like IP3R1, has been shown to increase during myeloid cell differentiation from hematopoietic precursors, whereas suppression of either PLSCR1 or IP3R1 expression impairs normal differentiation and maturation of these cells (12–16). This raises the possibility that the observed defects in growth factor-stimulated proliferation and maturation of cultured PLSCR1/− hematopoietic stem cells and the impaired granulopoiesis noted in PLSCR1-deficient mice are related in part to a diminished expression of cellular IP3 receptors and to consequent changes in regulated Ca2+ mobilization from intracellular stores as initiated through various growth factor receptors.

Our data suggest that in addition to the various biologic activities that have been attributed to PLSCR1 at the plasma membrane, this protein also has the potential to selectively alter gene transcription through its nuclear trafficking and binding to genomic DNA. Nuclear trafficking of PLSCR1 is observed in circumstances where palmitoylation of the polypeptide is prevented or precluded and is also observed in circumstances of transcriptionally induced increases in PLSCR1 expression, which may reflect deficient palmitoylation of the protein at high rates of translation (19). Although our data implicate IP3R1 as the target gene regulated by nuclear PLSCR1, it remains to be determined whether the transcription of other genes might also be affected in circumstances of its entry into the nucleus. For example, it was recently shown that the expression of a select subgroup of IFN-stimulated genes is suppressed in cells deficient in PLSCR1 and that this defective transcriptional response to IFN appears to underlie the diminished antiviral activity of IFN in PLSCR1-deficient cells (17). As was noted, PLSCR1 is itself transcriptionally induced by IFN through an IFN-stimulated response element in exon 1, and under circumstances of its induction by IFN, prominent nuclear trafficking of the protein has been observed (11, 19).

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