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Phospholipid Scramblase 1 Potentiates the Antiviral Activity of Interferon

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Phospholipid scramblase 1 (PLSCR1) is an interferon (IFN)- and growth factor-inducible, calcium-binding protein that either inserts into the plasma membrane or binds DNA in the nucleus depending on its state of palmyitoylation. In certain hematopoietic cells, PLSCR1 is required for normal maturation and terminal differentiation from progenitor cells as regulated by select growth factors, where it promotes recruitment and activation of Src kinases. PLSCR1 is a substrate of Src (and Abl) kinases, and transcription of the PLSCR1 gene is regulated by the same growth factor receptor pathways in which PLSCR1 potentiates afferent signaling. The marked transcriptional upregulation of PLSCR1 by IFNs led us to explore whether PLSCR1 plays an analogous role in cellular responses to IFN, with specific focus on antiviral activities. Accordingly, human cells in which PLSCR1 expression was decreased with short interfering RNA were rendered relatively insensitive to the antiviral activity of IFNs, resulting in higher titers of vesicular stomatitis virus (VSV) and encephalomyocarditis virus. Similarly, VSV replicated to higher titers in mouse *PLSCR1*$^{-/-}$ embryonic fibroblasts than in identical cells transduced to express PLSCR1. PLSCR1 inhibited accumulation of primary VSV transcripts, similar to the effects of IFN against VSV. The antiviral effect of PLSCR1 correlated with increased expression of a subset of IFN-stimulated genes (ISGs), including ISG15, ISG54, p56, and guanylate binding proteins. Our results suggest that PLSCR1, which is itself an ISG-encoded protein, provides a mechanism for amplifying and enhancing the IFN response through increased expression of a select subset of potent antiviral genes.

Interferons (IFNs) are the principal cytokines responsible for mediating innate immunity against viral infections (7). How IFNs establish an antiviral state in cells has been a subject of investigation since their discovery (21). Nevertheless, mechanisms of IFN action against viral infections remain incompletely understood. IFN antiviral studies have largely focused on several types of IFN-stimulated genes (ISGs), including the double-stranded RNA (dsRNA)-activated protein kinase (PKR), human myxovirus resistance proteins (Mx), 2′,5′-oligoadenylate synthetase (OAS) and its effector protein RNase L, ISG56 (p56), dsRNA-specific adenosine deaminase, and guanylate binding proteins (GBP) (35). Given the critical role of innate immunity in survival from infections, it is not surprising that the antiviral action of IFNs is complex and involves multiple overlapping or related pathways. For instance, mice that are triply deficient for RNase L, PKR, and Mx1 are nevertheless able to mount a substantial, residual IFN antiviral response (48). Therefore, identification of all of the antiviral ISGs is an important step toward a more complete appreciation and understanding of innate immunity. In this regard, within the past several years, global gene expression profiles from IFN-treated cells, obtained by DNA microarrays, have expanded the number of known ISGs from about 33 to >200 (12, 13).

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Phospholipid scramblase 1 (PLSCR1) is a novel ISG identified as such by way of DNA microarray analysis and confirmed by detailed analysis of the PLSCR1 promoter (12, 49, 50). In fact, PLSCR1 is highly induced by IFN-α, -β, and -γ and also by various growth factors, including epidermal growth factor (EGF), stem cell factor, and granulocyte colony-stimulating factor (30, 51). PLSCR1 is a multiply palmitoylated, lipid-raft-associated endofacial plasma membrane protein, with a proline-rich cytoplasmic domain containing several SH3 and WW domain binding motifs (38). PLSCR1 is proposed to accelerate bidirectional movement of plasma membrane phospholipids during conditions of elevated calcium (50). Transmembrane movement of phospholipids in response to calcium, however, is unaffected by either IFN treatment or PLSCR1 deletion (14, 49, 51).

Although the precise biologic function(s) of PLSCR1 and its related isoforms PLSCR2 to 4 remain to be determined (38), recent studies provide intriguing evidence of a role in cell signaling, maturation, and apoptosis. For instance, proliferation and terminal differentiation of certain hematopoietic stem cells (granulocyte precursor) populations is impaired in PLSCR1-null mice (51), and in both monocytic and granulocytic lineages, expression of this protein markedly increases with terminal differentiation into polymorphonuclear leukocytes or macrophages. Conversely, mutations affecting murine PLSCR1 have been associated with a leukemogenic phenotype, which is reversed upon expression of the wild-type (full-length) protein (24, 25). PLSCR1 suppressed ovarian carci-

noma in an animal model (37), and elevated expression of PLSCR1 has been shown to be required for normal myeloid differentiation (51). Finally, there is recent evidence that the level of expression of this protein correlates with overall survival in acute myelogenous leukemia (46). PLSCR1 is phosphorylated by select protein kinases, including Abl and Src, tyrosine kinases that participate in multiple growth factor receptor signaling pathways (30, 32, 41). Tyrosine phosphorylation of PLSCR1 by c-Src occurs in response to growth factors such as EGF, resulting in association of phosphorylated PLSCR1 with Shc and the activated EGF receptor complex (30). In the absence of PLSCR1, the activation of c-Src kinase through EGF receptor (and related receptors) is markedly attenuated, suggesting that PLSCR1 plays a role in growth factor-dependent recruitment or activation of c-Src kinase, potentially through its interaction in membrane lipid rafts (30, 40). Palmitoylation of PLSCR1 is required for insertion into the plasma membrane (44). However, when palmitoylation does not occur, the importin α/β nucleopore transport system has recently been shown to import PLSCR1 into the nucleus where it binds DNA (6, 44). Accordingly, newly synthesized PLSCR1 appeared in nuclei after IFN induction of PLSCR1 in the human ovarian carcinoma cell line, Hey1B (44). PLSCR1 is the only member of the PLSCR family thus far shown to be inducible by IFNs. These findings raise the possibility that PLSCR1 may contribute to the antiviral effects of IFNs by affecting viral penetration, IFN-stimulated cell signaling pathways at the plasma membrane, the transcription of antiviral genes in the nucleus, and/or by directly blocking specific stages in the viral replication cycle. To determine the involvement of PLSCR1 in the IFN-induced antiviral state, we have compared viral replication in wild-type and PLSCR1$^{-/-}$ mouse cells as well as in human cells in which PLSCR1 levels were decreased with short interfering RNA (siRNA). Our results demonstrate a marked suppression of viral replication by PLSCR1 which is accompanied by the enhanced expression of a specific subset of antiviral ISGs.

MATERIALS AND METHODS

Cell lines. The methods for establishment of mouse embryonic fibroblasts (MEFs) from C57BL/6 × SVev129 mice were previously described (30). Briefly, primary MEFs were isolated from embryos of PLSCR1$^{-/-}$ mice (51) or wild-type mice and immortalized by transfection with plasmid simian virus 40 large T antigen cDNA-pSV2 (KO1, PLSCR1$^{-/-}$, and wild-type cells). Mouse PLSCR1 (mPLSCR1) cDNA was cloned into a modified murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescence protein (GFP) vector, MSCV-IRES-GFP (derived from a plasmid generously provided by Ruibao Ren, Brandeis University), and transfected into the packaging cell line, PT-67 (BD Clontech), to yield infectious virus. mPLSCR1-MSCV-IRES-GFP was constructed as follows: v-abl-MSCV-IRES-GFP was digested with EcoRI and BamHI to remove v-abl cDNA. An SfiI cutting site (GGCCGCCTCGGCC) was inserted into the multiple cloning site of the MSCV-IRES-GFP vector by PCR-mediated insertion. mPLSCR1 cDNA was cloned into the EcoRI and SfiI sites of the modified MSCV-IRES-GFP vector. The KO1 MEFs were infected with MSCV-mPLSCR1-IRES-GFP virus to generate KI cells, or control MSCV-IRES-GFP virus was used to infect KO1 cells to generate KO2 cells. Infected cells were sorted by flow cytometry with GFP as an indicator to collect cells with similar expression levels of GFP. The expression of PLSCR1 was confirmed by Western blotting with monoclonal antibody against mPLSCR1(1A8) (30). The human ovarian carcinoma cell line Hey1B (a gift from Alexander Marks, University of Toronto, and Yan Xu, Cleveland Clinic) (4) and mouse L929 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin-penicillin and 10% heat-inactivated fetal bovine serum (FBS).

Hey1B cells stably expressing siRNA were established as described previously (10). Plasmid pSUPER$_{hPLSCR1}$ was generated by cloning a 19-nucleotide sequence (beginning 92 nucleotides from the translation start site in the human PLSCR1 [hPLSCR1] mRNA) separated by a spacer from its reverse complement as a BglII/HindIII fragment (synthesized at Invitrogen) into the pSUPER vector, which directs synthesis of an RNA from the H1-RNA promoter that is processed in the cell to siRNA (10). The sequences for primers of siRNA of hPLSCR1 are 5′-GAT-CCC-CGG-ACC-TCC-AGG-ATA-TAG-TGT-TCA-AGA-GAC-ACT-ATA-TCC-TGG-AGG-TCC-TTT-TTG-GAA-A-3′ and 3′-GG-GCC-TGG-AGG-TCC-TAT-ATC-ACA-AGT-TCT-CTG-TGA-TAT-AGG-ACC-TCC-AGG-AAA-AAC-CTT-TTC-GA-5′. The sequences for primers of the mismatch control are 5′-GAT-CCC-CGG-AC<u>G</u>-TCC-<u>T</u>GG-AT<u>T</u>-TAG-TGT-TCA-AGA-GAC-ACT-A<u>A</u>A-TCC-<u>A</u>GG-A<u>C</u>G-TCC-TTT-TTG-GAA-A-3 and 3′-GG-GCC-TG<u>C</u>-AGG-<u>A</u>CC-TA<u>A</u>-ATC-ACA-AGT-TCT-CTG-TGA-T<u>T</u>T-AGG-<u>T</u>CC-T<u>G</u>C-AGG-AAA-AAC-CTT-TTC-GA-5′ (mismatched nucleotides are underlined). The oligonucleotides were annealed by incubation in 100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), and 2 mM magnesium acetate at 95°C for 4 min and then at 70°C for 10 min. The reaction mixtures were slowly cooled to 4°C, and annealed oligonucleotides were phosphorylated with T4 polynucleotide kinase at 37°C for 30 min and incubated at 70°C for 10 min. Ligation of the oligonucleotides was to pSUPER digested with BglII and HindIII. The vectors containing siRNA to hPLSCR1, the 3-base mismatch control, and empty vector were each cotransfected with plasmid pBABE containing a puromycin resistance gene (10) into Hey1B cells by using Lipofectamine 2000 (Invitrogen). Stable cell lines were selected by continuous culturing in media containing 2 μg of puromycin per ml. PLSCR1 expression levels in cell lines were determined on Western blots probed with rabbit anti-hPLSCR1 peptide 306-318 antibody (50) and anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked (Cell Signaling) and β-actin monoclonal antibodies (Sigma Co.). Forty-seven clones expressing siRNA to PLSCR1 were isolated and screened by Western blot assays, of which about 20 clones had PLSCR1 protein levels that were ≤2-fold that of untreated parental cells. The siRNA clone that had the lowest level of PLSCR1 was used in these studies. In addition, individual clones containing the empty vector and the vector expressing the mismatch siRNA were isolated and determined by Western blotting to have PLSCR1 levels that were similar to those of the parental cells.

VSV purification and infections. Wild-type vesicular stomatitis virus (VSV) and an M protein late-budding domain or PY motif mutant (AAPA) (both were the Indiana strain) were propagated in BHK-21 cells (17). VSV was either from infected cell supernatant or was purified by sucrose gradient sedimentation (3) as indicated in the text. Briefly, virus in culture medium was pelleted by ultracentrifugation at 80,000 × *g* in a Beckman Rotor SW 41 or SW 28 for 120 min at 4°C. Virus pellets were suspended in phosphate-buffered saline (PBS) for 16 h at 4°C, loaded onto 0 to 40% sucrose gradients in 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, and 0.5 mM EDTA, and centrifuged at 35,000 × *g* in a Beckman rotor SW 41 for 90 min. The clear, white layer containing virus was collected and suspended in PBS at 4°C overnight, and the purified virus was stored at −70°C. All virus titers were determined by plaque assay (45) on soft agar overlays of L929 cells in six-well plates for incubation at 37°C for 1 to 2 days.

VSV infections were done after seeding cells in six-well plates (at 3×10^5 to 4×10^5 cells per well) and incubating them at 37°C in 5% CO_2 overnight. Cells were washed once with PBS and infected with a 0.1 multiplicity of infection (MOI) of VSV in FBS-free DMEM (Invitrogen) for 1 h followed by replacement of media with DMEM–10% FBS for different periods of time as indicated in the text. Cells were lysed with buffer containing 1% Triton X-100, 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% sodium deoxycholate, and 10 ng of leupeptin per ml, and extracts were centrifuged at 16,000 × *g* for 20 min. Media from infected cells were assayed for virus by plaque assays or for viral proteins in media of infected cells or supernatant of the cell lysates by Western immunoblot assays.

Immunoblots. Rabbit antibody 4720 against N-terminal residues 1 to 12 of mPLSCR1 (41) and rabbit antibody against C-terminal residues 306 to 318 of hPLSCR1 (50) were previously described (each are rabbit antipeptide antisera that are affinity purified on the peptide and thus used as affinity-purified IgG). Other antibodies used were rabbit anti-N protein of VSV (8), rabbit anti-L protein of VSV raised against a synthetic peptide corresponding to amino-terminal residues 5 to 19 of the L protein (29), mouse monoclonal anti-VSV M protein (a gift from D. S. Lyles, Winston-Salem, N.C.), mouse monoclonal anti-VSV G protein (no. 1667351; Roche), rabbit anti-p56 (a gift from Ganes Sen, Cleveland, Ohio) (16), mouse monoclonal anti-p15 (from Ernest Borden, Cleveland, Ohio) (11), rabbit anti-mGBP-2 (from Deborah Vestal, Toledo, Ohio) (42), and monoclonal anti-β-actin (catalog number A-5441; Sigma Co.). Proteins (30 to 60 μg) in cell extracts or 25 μl of medium from virus-infected cells was separated on 8 to 12% polyacrylamide–sodium dodecyl sulfate (SDS) gels and transferred onto Immobilon-P transfer membranes (Millipore Co.). Blots were

blocked with PBS containing 0.07% Tween (PBS-T) and 5% fat-free dried milk for 1 h and then incubated with primary antibodies in the same blocking buffer at room temperature for 2 h or at 4°C for 16 h. The blots were washed three times with PBS-T. After a 1-h incubation of blots with secondary antibody, anti-mouse IgG-HRP, or anti-rabbit IgG-HRP (Cell Signaling Co.) and four washes with PBS-T, protein bands were visualized with enhanced chemiluminescence detection reagents (Amersham Co.). Protein amounts were estimated with the NIH Image (version 1.61) computer program.

VSV adsorption and penetration. The ^{35}S-labeled VSV was prepared from 2×10^7 BHK-21 cells infected with VSV (MOI = 0.1) in methionine-free DMEM (Invitrogen Co.) in the absence of serum for 1 h and washed with PBS. Methionine-free DMEM containing both 3 μg of actinomycin D per ml and 1.4 mCi of [^{35}S]methionine was added to the cells, and cells were incubated for 24 h. The ^{35}S-labeled VSV in the media was purified by sucrose gradient sedimentation as described above. KO and KI cells were plated 1 day prior to infection in 12-well plates with 6×10^4 cells per well and incubated with purified ^{35}S-labeled VSV (MOI = 4) in FBS-free DMEM at 37°C for 1 h. After cells were washed twice with PBS, complete DMEM with 10% FBS was added and cells were incubated for 1.5 h. Lysis buffer was added to the cells after the cells were washed three times with PBS. The cell lysates were centrifuged at 16,000 × *g* for 20 min, the protein extracts were fractionated on 10% polyacrylamide–SDS gels, and an autoradiogram was prepared from the dried gels. Radiolabeled viral protein amounts were estimated by using NIH Image (version 1.61).

Primary VSV mRNA transcript accumulation. VSV N mRNA accumulation owing to primary transcription was measured as described previously (8). KO2 and KI cells were pretreated with 1,000 U of recombinant human IFN-α A/D (Hoffmann LaRoche, Inc.) per ml for 16 h and washed with PBS once. Cycloheximide (10 μg/ml) in DMEM with 10% FBS was added to the cells for 2.5 h followed by washing with PBS. Purified VSV at an MOI of 0.5 with FBS-free DMEM and 10 μg of cycloheximide/ml was added to the cells for 1 h. After removing the virus, cells were incubated with 10% FBS–DMEM with cycloheximide (10 μg per ml) for 3, 5, and 8 h before cells were harvested for RNA extraction with Trizol (Invitrogen). RNA (10 μg) was separated on 1.2% agarose–formaldehyde gels, transferred to a Hybond-N$^+$ membrane (Amersham Biosciences), and cross-linked with UV. The blots were incubated with ULTRAhyb (Ambion) hybridization buffer at 42°C for 4 h, prior to the addition of ^{32}P-cDNA, encoding the VSV N protein (8, 20, 33), which was labeled by using the Prime-a-Gene system (Promega) at 42°C for 16 h. The autoradiograms of the blots were prepared after washing four times at 50°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS. The blots were stripped and reprobed with ^{32}P-labeled β-actin cDNA.

EMCV infections. Encephalomyocarditis virus (EMCV) (a gift of I. Kerr, London, England) was propagated by infecting L929 cells, collecting the cell supernatants, and clearing by centrifugation at 16,000 × *g* for 20 min at 4°C. Virus titers were determined by plaque assays with L929 cells. EMCV was used to infect cells at an MOI of 0.01, in FBS-free DMEM for adsorption (1 h), which was then replaced with 10% FBS–DMEM. The media containing progeny virus were collected after 24 and 40 h.

Gene expression profiling by use of custom cDNA microarrays. Cells stably expressing siRNA to hPLSCR1 and mismatch control and vector control cells were plated in triplicate at 4×10^5 cells per well in six-well plates and incubated at 37°C with 5% CO_2 for 16 h. Cells were incubated in the absence and presence of 1,000 U of human IFN-β (Interpharma) per ml for 8 h and washed with PBS. Total RNA was extracted with Trizol reagent while identically treated cells were harvested for determining PLSCR1 levels.

Array construction. The array used in this study comprised a subset of sequence-verified cDNA clones from the Research Genetics, Inc., 40,000-clone set representing 950 genes containing adenylate-uridylate-rich elements and 18 genes potentially involved in AU-directed mRNA decay as previously described (15), 855 ISGs representing an expansion of a previously described clone set containing confirmed and candidate genes stimulated by IFNs in diverse cell types (13), 288 genes responsive to the viral analog poly(I-C), and 85 housekeeping genes. DNA preparation and slide printing were as previously described except for the use of 40% dimethyl sulfoxide in place of 1.5× SSC as the printing solution (15).

Target RNA preparation. Target RNA was generated in a T7 polymerase-based linear amplification reaction based on a modified version of a published protocol (43). Two micrograms of total RNA and 5 pmol of T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′] in a total volume of 5.5 μl was incubated at 70°C for 10 min and chilled on ice. For first-strand cDNA synthesis, the annealed RNA template was incubated for 1 h at 42°C in a 10-μl reaction mixture containing first-strand buffer (Invitrogen), 10 mM dithiothreitol, 1 U of anti-RNase (Ambion) per μl, 500 μM deoxynucleoside triphosphates, and 2 U of Superscript II (Invitrogen) per μl. Second-strand synthesis was for 2 h at 16°C in a total reaction volume of 50 μl containing first-strand reaction products, second-strand buffer (Invitrogen), 250 μM deoxynucleoside triphosphates, 0.06 U of DNA ligase (Ambion) per μl, 0.26 U of DNA polymerase I (New England Biolabs) per μl, and 0.012 U of RNase H (Ambion) per μl followed by the addition of 3.3 U of T4 DNA polymerase (3 U per μl; New England Biolabs) and a further 15 min of incubation at 16°C. Second-strand reaction products were purified by phenol-chloroform-isoamyl alcohol extraction in Phaselock microcentrifuge tubes (Eppendorf) according to the manufacturer's instructions and ethanol precipitated. In vitro transcription was performed by using the T7 megascript kit (Ambion) according to a modified protocol in which purified cDNA was combined with 1 μl (each) of 10× ATP, GTP, CTP, and UTP and 1 μl of T7 enzyme mix in a 10-μl reaction volume and incubated for 9 h at 37°C. Amplified RNA was purified by using the Rneasy RNA purification kit (Ambion) according to the manufacturer's instructions.

RNA labeling. Cy3- or Cy5-labeled cDNA was prepared by indirect incorporation. Two micrograms of amplified RNA, 1 μl of dT_{12-18} primer (1 μg per μl; Invitrogen), 2.6 μl of random hexanucleotides (3 μg per μl; Invitrogen), and 1 μl of anti-RNase (Ambion) were combined in a reaction volume of 15.5 μl and incubated for 10 min at 70°C. Reverse transcription was for 2 h at 42°C in a 30-μl reaction mixture containing annealed RNA template, first-strand buffer, 500 μM (each) dATP, dCTP, and dGTP, 300 μM dTTP, 200 μM aminoallyl-dUTP (Sigma), 10 mM dithiothreitol, and 12.7 U of Superscript II per μl. For template hydrolysis, 10 μl of 0.1 M NaOH was added to the reverse transcription reaction mixture and the mixture was incubated for 10 min at 70°C, allowed to cool at room temperature for 5 min, and neutralized by the addition of 10 μl of 0.1 M HCl. cDNA was precipitated at −20°C for 30 min after the addition of 1 μl of linear acrylamide (Ambion), 4 μl of 3 M sodium acetate (pH 5.2), and 100 μl of absolute ethanol, and then resuspended in 5 μl of 0.1 M $NaHCO_3$. For dye coupling, the contents of 1 tube of *N*-hydroxysuccinimide ester containing Cy3 or Cy5 dye (product no. PA25001 and PA25002; Amersham Biosciences) was dissolved in 45 μl of dimethyl sulfoxide. Five microliters of dye solution was mixed with the cDNA and incubated for 1 h in darkness at room temperature. Labeled cDNA was purified on a QIAquick PCR purification column (QIAGEN) according to the manufacturer's instructions. Eluted cDNA was dried under a vacuum and resuspended in 30 μl of Slidehyb II hybridization buffer (Ambion). After 2 min of denaturation at 95°C, the hybridization mixture was applied to the microarray slide under a coverslip. Hybridization proceeded overnight in a sealed moist chamber in a 55°C water bath. Posthybridization, slides were washed successively for 5 min each in 2× SSC–0.1% SDS at 55°C, then in 2× SSC at 55°C, and finally, in 0.2× SSC at room temperature.

Acquisition and normalization of data. Data were acquired with a GenePix 4000B laser scanner and GenePix Pro, version 5.0, software as previously described (15). Raw data were imported into GeneSpring, version 6.0, software (Silicon Genetics) and normalized based on the distribution of all values with locally weighted linear regression before further analysis.

RESULTS

PLSCR1 contributes to antiviral activities of IFNs. To investigate the involvement of PLSCR1 in the IFN-induced antiviral state, PLSCR1 levels were stably decreased in the human ovarian carcinoma cell line Hey1B by using an siRNA approach. An siRNA against a PLSCR1 mRNA target site, 92 to 110 nucleotides 3′ to the translation start site, was generated from a DNA sequence cloned into plasmid pSUPER (10). As a control, siRNA was generated with 3 mismatched nucleotides to the same PLSCR1 sequence and with compensatory base changes in the opposite strand to maintain base pairing. In addition, cells containing the empty plasmid vector were also used as controls. Extracts of stable clones obtained by drug selection were screened for PLSCR1 expression in Western blots probed with antibodies to both PLSCR1 and β-actin. Cell lines with empty vector (vector control) and with the mismatch siRNA showed similar basal levels of PLSCR1 that increased markedly with IFN-β treatment (Fig. 1A, lanes 1 to 4). In contrast, the PLSCR1 siRNA plasmid reduced the basal expression of PLSCR1 by about 10-fold (relative to basal expression in untreated controls). Furthermore, the IFN-induced level of

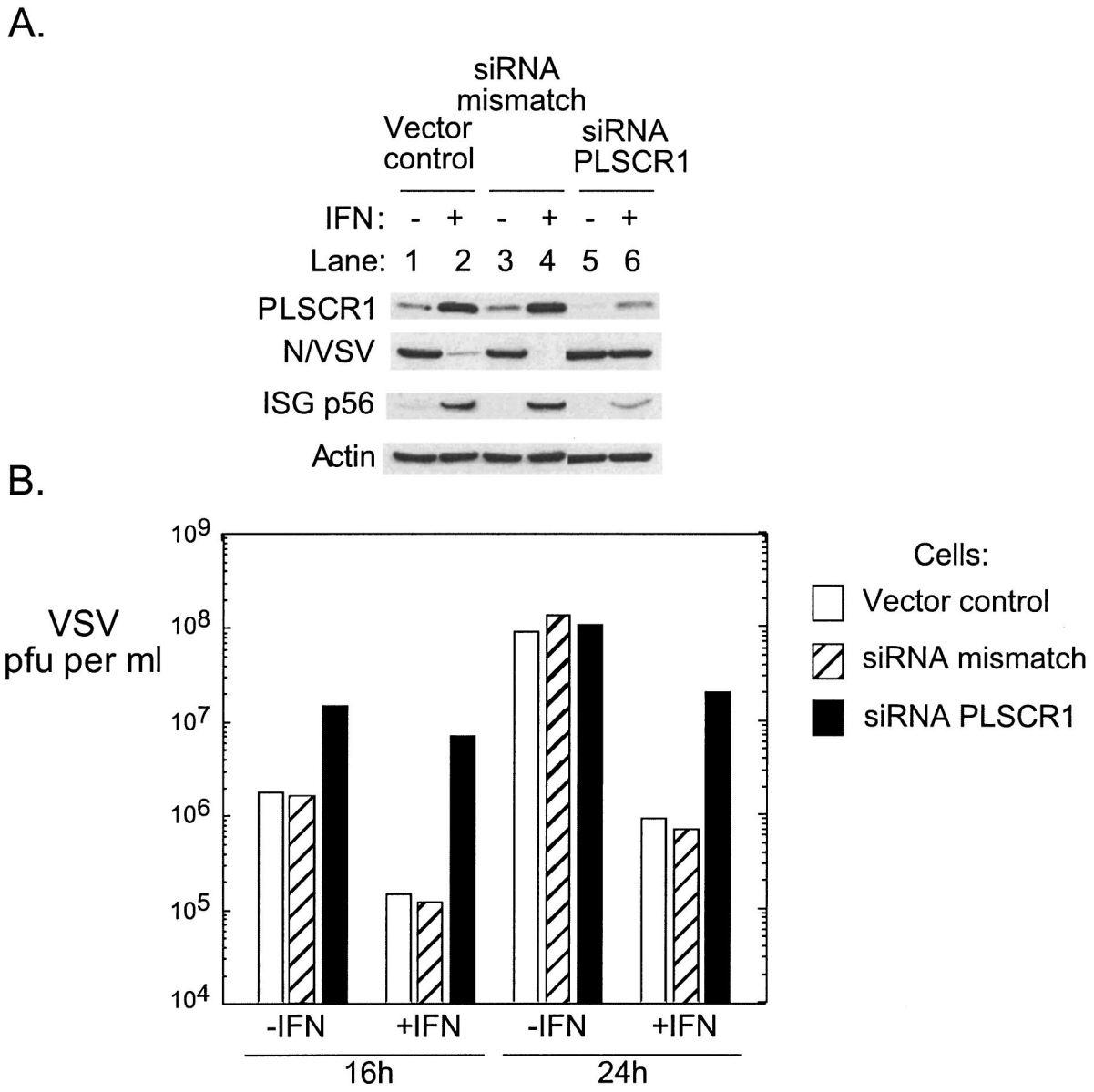

FIG. 1. Decreasing levels of PLSCR1 with siRNA suppresses the anti-VSV activity of IFN in human Hey1B cells. Hey1B cells stably transfected with pSUPER lacking insert (vector control) or pSUPER expressing the siRNA mismatch control or siRNA to PLSCR1 were incubated with or without human IFN-β (1,000 U per ml) for 8 h and were then infected with purified VSV at an MOI of 0.1 for 16 and 24 h. (A) Levels of hPLSCR1, VSV N protein, p56, and β-actin were determined at 24 h postinfection from cell extracts in Western blots probed with antibodies. (B) VSV yields were determined by plaque assays after combining media from triplicate cultures of infected cells preincubated in the presence (+) or absence (−) of IFN-β (as indicated).

PLSCR1 was also suppressed severalfold through PLSCR1-specific siRNA compared to the IFN-stimulated controls not containing the PLSCR1 siRNA plasmid (Fig. 1A, top panel, compare lanes 2, 4, and 6).

To investigate the potential antiviral effect of PLSCR1, cells were incubated in the presence or absence of IFN-β (1,000 U per ml) for 8 h and subsequently infected with the VSV Indiana strain at an MOI of 0.1 (a member of the *Rhabdoviridae* family of enveloped, nonsegmented negative-strand RNA viruses). Levels of VSV N protein and p56 (encoded by an ISG) were determined at 24 h postinfection (Fig. 1A). VSV N protein amounts were greatly reduced by IFN-β in both the vector control and siRNA mismatch-expressing cells (Fig. 1A, lanes 1 to 4). Remarkably, however, N protein levels appeared only slightly decreased after IFN-β treatment of cells with siRNA to PLSCR1 (Fig. 1A, lane 6). Furthermore, reducing the PLSCR1 levels resulted in increases of VSV yields (about 1 $\log_{10}$ unit) by 16 h but not at 24 h postinfection, possibly indicating that PLSCR1 was causing a delay in the replication cycle (Fig. 1B). The antiviral effect of IFN-β at both 16 and 24 h postinfection, however, was substantially impaired when PLSCR1 levels were decreased (Fig. 1B). At 24 h postinfection, IFN-β reduced

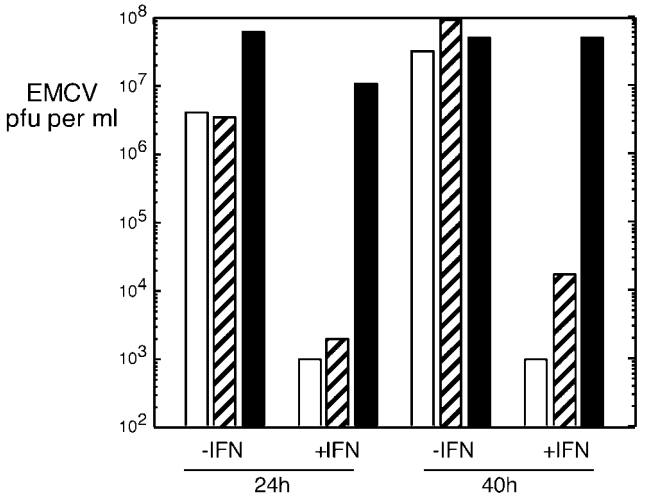

FIG. 2. PLSCR1 suppresses EMCV replication in Hey1B cells. Hey1B cells stably transfected with empty vector (vector control, white bars) or with vector expressing mismatched siRNA (hatched bars) or PLSCR1 siRNA (black bars) were treated with IFN-β (1,000 U per ml) for 8 h and infected with EMCV (MOI of 0.01) for 24 and 40 h. Viral titers from combining media of triplicate infected cultures of cells were determined by plaque assays. +, present; −, absent.

VSV yields by about 200-fold in the vector control and siRNA mismatch cells, whereas the IFN effect was only 5-fold in the cells expressing PLSCR1 siRNA (Fig. 1B). To monitor IFN induction of gene expression, levels of p56 protein were measured with a monoclonal antibody. IFN-β treatment of both the empty vector and mismatch siRNA control cells induced p56 to severalfold-higher levels than in the IFN-treated PLSCR1 siRNA cells (Fig. 1A, lanes 2, 4, and 6). The effect of PLSCR1 on p56 expression required the addition of exogenous IFN and was not observed with virus alone. These results suggested a possible contribution of PLSCR1 to IFN induction of gene expression. There was no increase of p56 expression in the control siRNA-expressing cells (Fig. 1A, compare lanes 1 and 3), suggesting that pSUPER expression of siRNA in these cells did not produce an off-target, nonspecific effect on ISG expression (9, 39). This conclusion was supported by gene array results (see below).

To determine whether the antiviral activity of PLSCR1 was specific for VSV, growth of EMCV (a member of the *Picornaviridae* family of nonenveloped, positive-strand RNA viruses) was compared in the different cell lines (Fig. 2). The Hey1B cells were incubated in the presence or absence of human IFN-β (1,000 U per ml) for 8 h and then infected with EMCV at an MOI of 0.01. In the absence of IFN, suppression of basal PLSCR1 expression by specific siRNA resulted in an about 10-fold increase in viral replication at 24 h postinfection,

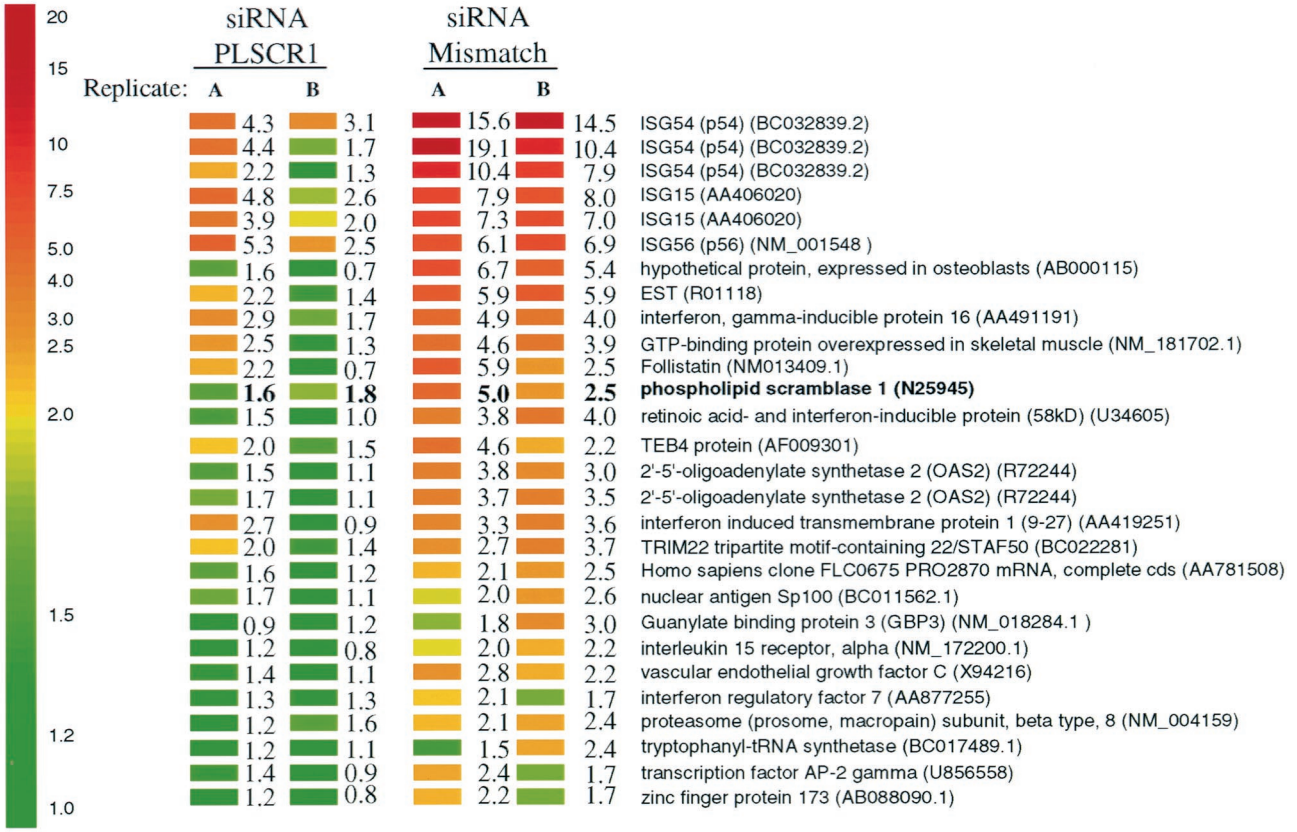

FIG. 3. PLSCR1 enhances expression of a set of ISGs as determined in DNA microarrays. Hey1B cells expressing siRNA mismatch or siRNA to PLSCR1 were incubated with or without IFN-β (1,000 U/ml) for 8 h. Gene array results are from RNA samples isolated from triplicate cultures of IFN-treated or control cells. Numbers represent increases (*n*-fold) in RNA levels after IFN treatment.

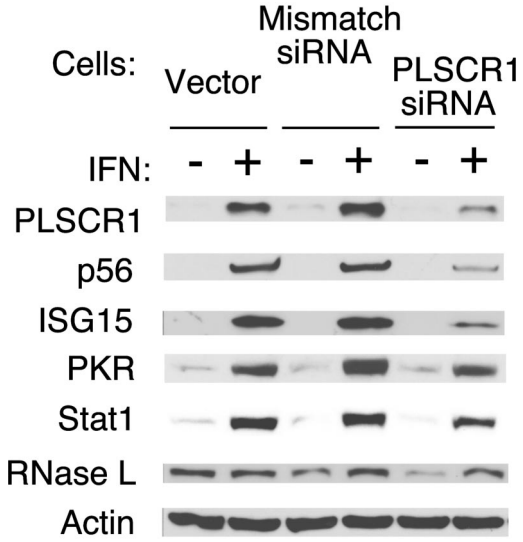

FIG. 4. PLSCR1 enhances the expression of a subset of ISGs as determined by Western immunoblots. Hey1B cells containing empty vector (vector) or expressing siRNA mismatch or siRNA to PLSCR1 were incubated with (+) or without (−) IFN-β (1,000 U/ml) for 16 h. Levels of proteins (indicated) were determined by probing Western blots of cell extracts with specific antibodies (see Materials and Methods).

although no effect was seen at 40 h, suggesting a small delay in viral replication due to PLSCR1. In contrast to these small effects in the absence of IFN, in cells pretreated with IFN, the suppression of PLSCR1 expression by specific siRNA eliminated most of the observed antiviral activity associated with addition of IFN (cf. black bars to controls in Fig. 2).

Reduced expression of ISGs in PLSCR1-deficient cells. Our findings suggest a general antiviral effect of PLSCR1 that appears related to marked enhancement of the cellular response to IFN. We therefore next compared mRNA profiles in untreated and IFN-β-treated cells by using a custom viral response cDNA microarray constructed with 855 candidate and confirmed ISGs, 950 AU-rich element genes, 288 dsRNA-stimulated genes, and 85 housekeeping genes, with the latter serving as mRNA controls (Fig. 3). Triplicate cultures of Hey1B vector control, mismatch siRNA, or PLSCR1 siRNA clones were treated with IFN-β at 8 h, and the RNA isolated from identically treated cultures was combined for microarray analysis. The experiment was independently performed twice (i.e., experiments A and B were both from RNA pools of triplicate, identically treated cultures). In addition, several of the ISGs were present at multiple positions on the array (indicated by multiple rows for the same gene in Fig. 3). Twenty-four genes were more-highly induced by IFN-β in the control cells expressing mismatched siRNA than in the cells expressing specific siRNA to PLSCR1. Twenty-one of these genes are previously identified ISGs. Three genes are newly identified ISGs from these experiments and are also AU-rich genes (hypothetical protein expressed in osteoblasts, TEB4, and transcription factor AP-2 gamma). ISG54, present at three locations on the array, was one of the most highly elevated ISGs associated with PLSCR1 expression. The average IFN induction of ISG54 was about fivefold greater in the control siRNA cells than in the PLSCR1 siRNA cells. The remaining 23 ISGs were induced 1.7- to >5-fold greater by IFN in the control siRNA cells than in the PLSCR1 siRNA-expressing cells. Our results suggest a contribution of PLSCR1, a known ISG (12, 49), to the IFN-stimulated expression of a limited subset of ISGs. However, because siRNA ablation of PLSCR1 was incomplete, the values obtained may underestimate the contribution of PLSCR1 to ISG expression. A decreased IFN induction of PLSCR1 itself was observed in the siRNA to PLSCR1 cells. PLSCR1 siRNA did not significantly affect expression of any of the 85 housekeeping genes serving as controls (data not shown).

To confirm the gene array results, immunoblot measurement of several different IFN-induced proteins was performed (Fig. 4). Deficient IFN induction of PLSCR1, p56, and ISG15 was observed; there was a small effect on Stat1 levels while PKR and RNase L amounts were essentially unaffected. The siRNAs by themselves did not induce ISG expression, as determined by both gene microarrays and Western blot assays (Fig. 4 and data not shown).

The IFN response is reduced in PLSCR1$^{-/-}$ MEFs. To perform studies in the complete absence of PLSCR1, PLSCR1$^{-/-}$

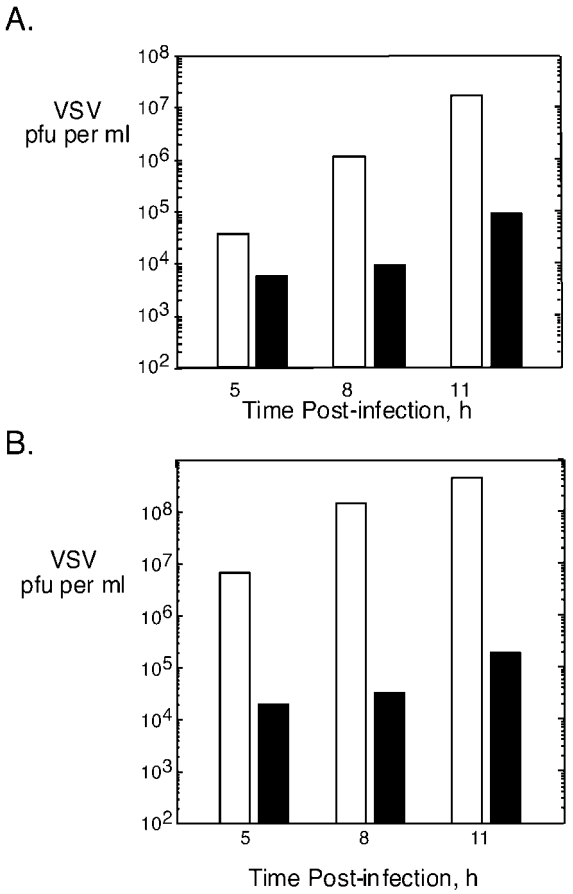

FIG. 5. VSV replicates to higher titers in MEFs lacking PLSCR1. (A) Wild-type (black bars) and PLSCR1$^{-/-}$KO1 (white bars) MEFs were infected with VSV at an MOI of 0.1. (B) PLSCR1$^{-/-}$ KO2 (white bars) MEFs and reconstituted, PLSCR1-expressing knock-in KI cells (black bars) were infected with VSV at an MOI of 0.1. At different times postinfection (*x* axes), virus was harvested. Viral yields, determined by plaque assays on indicator L929 cells, were from combined triplicate cultures of infected cells.

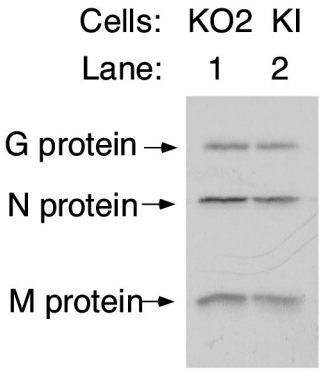

FIG. 6. Adsorption and penetration of ^{35}S-labeled VSV is unaffected by PLSCR1. KO2 (PLSCR1$^{-/-}$) and KI (PLSCR1 reconstituted) cells were infected with purified ^{35}S-VSV (MOI of 4) (see Materials and Methods). Cell-associated proteins were separated by SDS-polyacrylamide gel electrophoresis, and an autoradiogram of the dried gel was prepared. The positions of the VSV G, N, and M proteins are indicated (arrows).

MEFs immortalized with simian virus 40 large T antigen (KO1 cells) were utilized. VSV yields were increased up to about 100-fold in the KO1 cells compared to the wild-type cells (Fig. 5A). To rule out nonspecific effects owing to clonal variations, the KO1 cells were transfected with MSCV vector expressing PLSCR1 cDNA (KI cells for gene knock-in) or with an empty MSCV vector (KO2 cells). VSV yields were 3 to >4 $\log_{10}$ units higher in the KO2 cells than in KI cells (Fig. 5B). These results from KO cells deficient in PLSCR1 are consistent with results obtained with wild-type cells in which endogenous PLSCR1 expression was suppressed by siRNA, although the PLSCR1-related antiviral effect was even more apparent in the former, where PLSCR1 is completely absent.

Determining the stage(s) in the VSV replication cycle affected by PLSCR1. To examine how PLSCR1 affects VSV replication, several distinct stages in the viral life cycle were analyzed and compared in the KO2 and KI cells. Viral absorption and penetration were determined by infecting cells with purified ^{35}S-labeled VSV for 2.5 h and monitoring cell-associated proteins that originated from the input virus. After washing and lysing the infected cells, proteins were subjected to electrophoresis, transfer, and autoradiography (Fig. 6). Equivalent amounts of the ^{35}S-labeled VSV proteins, G (glycoprotein), N (nucleoprotein), and M (matrix protein), were observed associated with the KO2 and KI cells. These results suggest that PLSCR1 did not affect stages prior to viral penetration.

Primary viral transcript accumulation was monitored by measuring VSV N mRNA produced from the input genome in the presence of cycloheximide. This method relies on the fact that amplification of VSV RNA, but not primary transcription, requires ongoing protein synthesis. Previously, IFN was reported to suppress VSV replication at the level of viral primary transcription (5). In the present studies, IFN-α pretreatments effectively reduced primary viral transcript accumulation in both cell lines (Fig. 7). Furthermore, expression of PLSCR1 reduced N mRNA accumulation in either the absence or presence of prior IFN treatment. Therefore, both IFN and PLSCR1 suppressed VSV replication at the level of primary transcript accumulation. Accordingly, in cells infected in the absence of cycloheximide, VSV L, G, N, and M proteins were significantly more abundant in the media (from released virus) and from intact KO2 cells than were released virus and cell-associated virus of the KI cells (Fig. 8). An additional effect on viral protein synthesis is not ruled out by these findings (34).

To determine whether late stages in the virus replication cycles were affected by PLSCR1, release of progeny wild-type VSV and of a late-budding domain (PPPY to AAPA) M protein mutant virus into the media was compared in the KO2 and KI cells. The M protein mutation was previously observed to reduce viral release by about 1 to 2 $\log_{10}$ units (23). Similarly, the AAPA mutant form of M protein reduced viral yields by 45-fold in the KO2 cells and by 62-fold in the KI cells (Fig. 9). These results suggest that the anti-VSV effect of PLSCR1 does not depend on or require the late-budding PY domain in M protein but does not exclude the possibility that PLSCR1 may

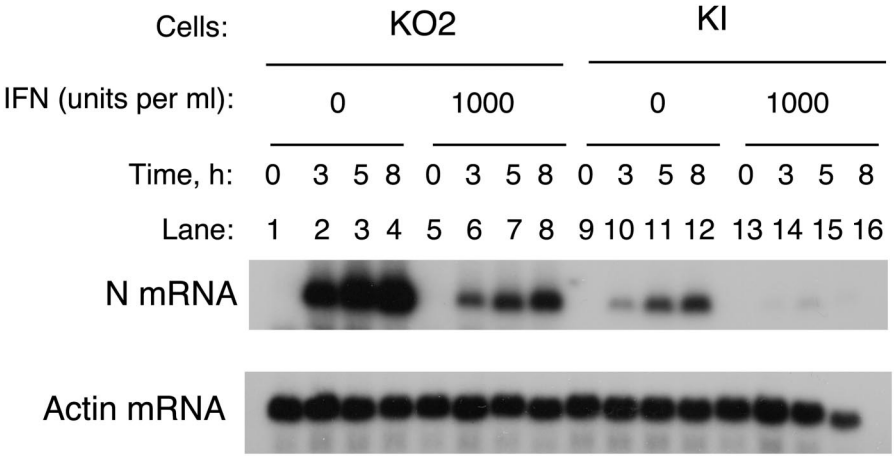

FIG. 7. PLSCR1 and IFN-α inhibit accumulation of primary VSV N transcripts. Cells were incubated with or without IFN-α A/D (1,000 U per ml) for 16 h followed by treatment with cycloheximide (3 μg/ml) for 2.5 h. Infections were with purified VSV (MOI of 0.5) for 0, 3, 5, and 8 h in the continuous presence of cycloheximide to prevent replication. The Northern blot was probed with ^{32}P-cDNA to the N gene of VSV and was normalized with a radiolabeled cDNA to β-actin.

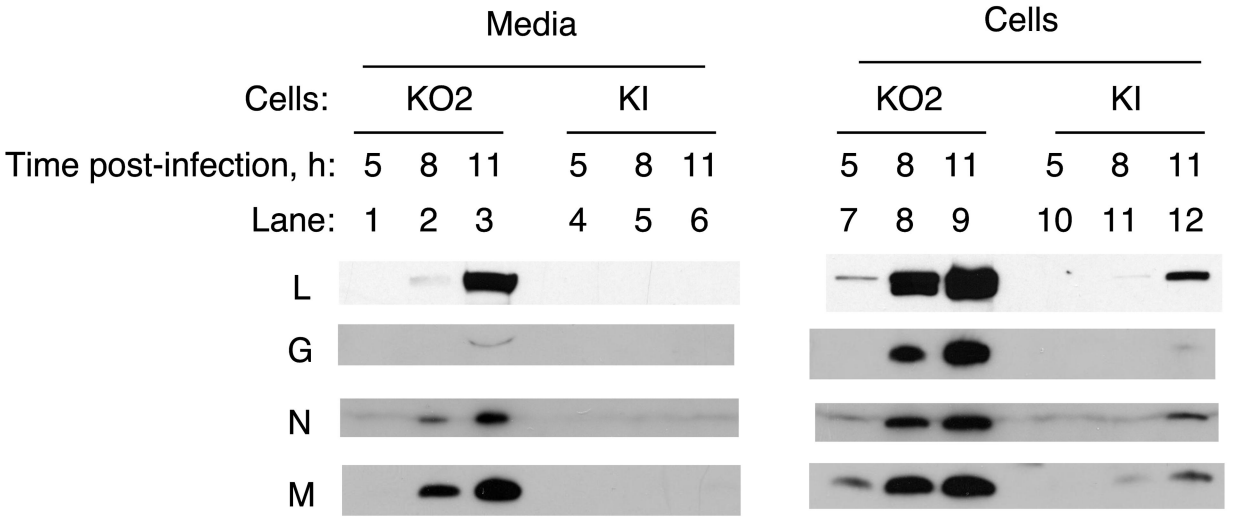

FIG. 8. VSV protein accumulation is reduced in cells expressing PLSCR1. KO2 and KI cells were infected with purified VSV at an MOI of 0.1 for 5, 8, and 11 h (as indicated). Levels of VSV proteins from released virus (Media) and associated with intact cells (Cells) were determined on Western blots probed with antibodies to the VSV L, G, N, and M proteins.

influence viral budding, for example, through effects on plasma membrane lipid organization or topology.

PLSCR1 expression was equivalent in the wild-type and KI cells and was absent in the KO1 and KO2 cells (Fig. 10 and data not shown). In the wild-type cells, PLSCR1 levels were constitutively elevated and were not further increased by IFN-α treatment, perhaps due to induction by growth factors in the serum (51) (Fig. 10, lanes 4 to 6). Cell-type-specific differences in basal levels of PLSCR1 may reflect inherent lineage or maturational differences in PLSCR1 expression as well as differing sensitivities of the cells to induction by growth factors or interferon. GBP-2 was induced by 10-fold-lower concentrations of IFN-α in the wild-type and KI cells than in the KO1 cells (Fig. 10) (42). However, IFN-induced levels of PKR and STAT1 were similar in the different cell lines. Basal levels of PKR, however, were modestly elevated in untreated confluent (24 h) cultures of PLSCR1-expressing cells compared with KO1 cells (Fig. 10, bottom, lanes 1, 4, and 7). These findings are consistent with the notion that PLSCR1 affects the expression of only a limited subset of ISGs.

DISCUSSION

Our results suggest that the expression of PLSCR1, an ISG, is required for maximal antiviral activity of IFN, and that this effect is mediated at least in part through potentiation of the expression of a select subset of ISGs with known or suspected antiviral activities. However, PLSCR1 is nonessential for IFN signaling because IFN strongly induces PKR and STAT1 in PLSCR1$^{-/-}$ cells (Fig. 10). Whereas the precise mechanism by which PLSCR1 exerts these selective effects on certain ISGs remains unresolved, it is of note that (i) PLSCR1 is a palmitoylated, plasma membrane protein known to partition into lipid rafts and implicated in regulating the organization of plasma membrane phospholipids (38, 40), (ii) deletion of PLSCR1 has been shown to alter afferent signaling and cellular response to a select group of cell surface growth factor receptors with specific effects on the activation of c-Src and potentially other protein kinases (30, 32, 41), (iii) in addition to transcriptional induction by IFN, PLSCR1 expression is upregulated through each of the growth factor receptor pathways that PLSCR1 gene deletion has been shown to attenuate afferent receptor signaling (30, 51), (iv) under conditions of transcriptional induction, PLSCR1 has been shown to traffic to both the plasma membrane and the nucleus, events that appear to be regulated through its palmitoylation (44), and (v) once in the nucleus, PLSCR1, an acidic polypeptide, is found tightly bound to DNA (6). Taken together, this suggests that the observed antiviral activity of PLSCR1 and its capacity to potentiate transcription of a select subset of ISGs reflects activities of this protein at the plasma membrane that potentially influence afferent signaling through the JAK/STAT kinase pathway (or accessory signaling pathways recruited downstream of the activated receptor), resulting in alteration of the

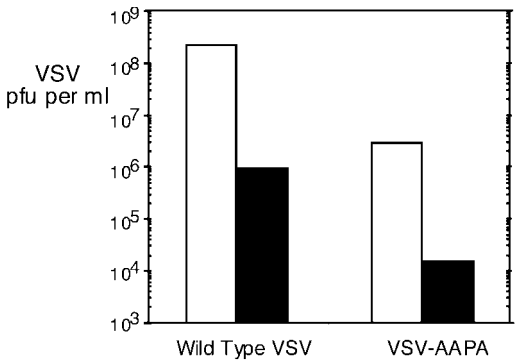

FIG. 9. Replication of VSV with a late-budding domain mutation (AAPA) in the M protein and wild-type VSV were similarly inhibited by PLSCR1. KO2 (white bars) and KI (black bars) cells were infected with wild-type VSV and VSV-AAPA mutant virus (MOI of 0.1) for 16 h. The viral yields in the media combined from three separate infections of cells were determined by plaque assays on indicator cells.

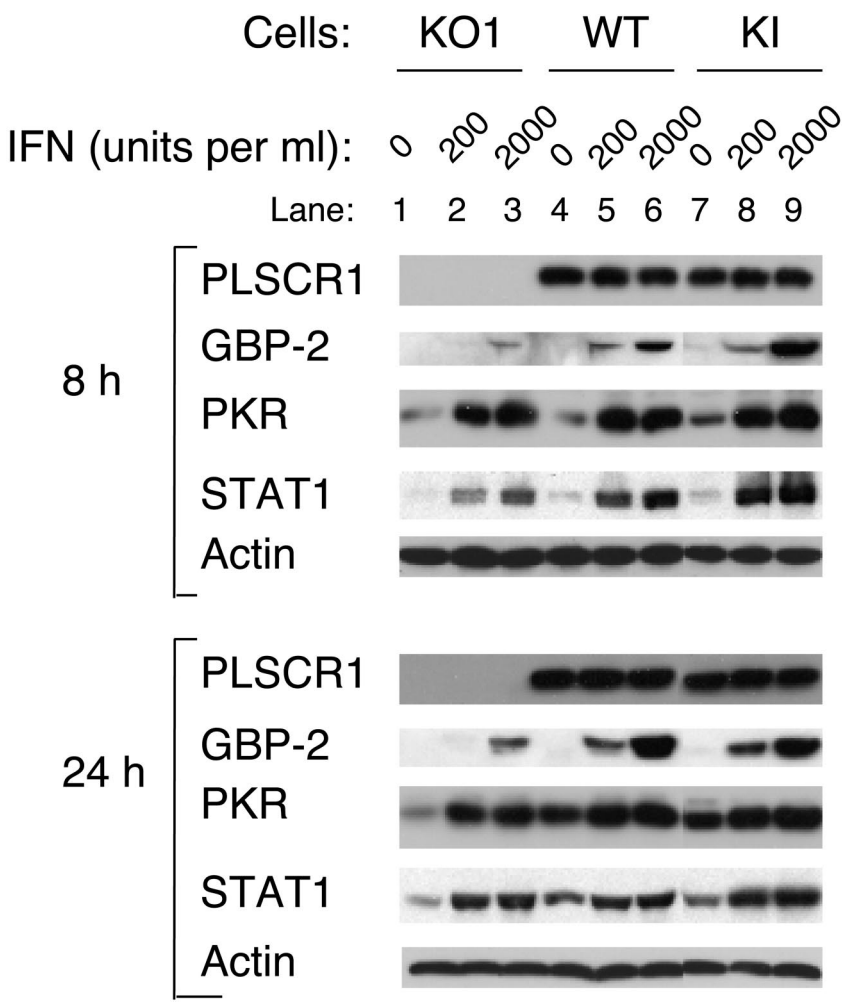

FIG. 10. IFN-induced and basal levels of PLSCR1, GBP-2, PKR, and Stat1 in IFN-treated and control MEFs that contain or lack PLSCR1. Cells were incubated for 8 or 24 h in the presence or absence of different concentrations of IFN-α A/D (as indicated). Cells harvested at 8 and 24 h were subconfluent and confluent, respectively. Western blots probed with antibodies to PLSCR1, GBP-2, PKR, and Stat1 are shown.

repertoire of activated transcription factors, and/or effects of nuclear PLSCR1 on the transcription of select ISGs.

Regarding the specific ISGs positively regulated by PLSCR1, the virus stress-inducible proteins p54 and p56 (encoded by ISG54 and ISG56, respectively) are related members of a protein family containing tetratricopeptide motifs (19). Protein p56 interacts with the protein synthesis initiation factor ε subunit of eukaryotic initiation factor 3 (eIF-3ε) and inhibits translation by interfering with the binding of eIF-2–GTP–Met-tRNA$_i$ (ternary complex) with eIF-3. Therefore, p56 has the ability to suppress translation of virus and host proteins. The functions of the other family members, p54, p58, and p60, are unknown. ISG15 contains two ubiquitin homology domains and is ligated to diverse proteins, including Jak1 and Stat1, and has been suggested to play a positive role in IFN signaling (26, 28). The NS1 protein of influenza B virus inhibits linkage of ISG15 to its target proteins, supporting an antiviral role for ISG15 (47). OAS2 is one of the upstream enzymes in the 2′,5′-oligoadenylates (2-5A)/RNase L antiviral pathway that synthesizes 2-5A in response to viral dsRNA. 2-5A activates RNase L, causing breakdown of viral and host RNA (36). RNase L$^{-/-}$ mice are partially deficient in the anti-EMCV effect of IFN-α (48). GBP-2 and GBP-3 are members of an IFN-induced gene family of at least five different GBPs (31, 42). GBP-1 was shown to inhibit replication of VSV and EMCV, but the mechanism is unknown (1). Expression of PLSCR1 was also associated with enhanced basal expression of PKR in confluent, but not in subconfluent, mouse cells (Fig. 10). Therefore, PKR may also contribute to the observed antiviral effects of PLSCR1. PKR is activated by viral dsRNA to phosphorylate translation initiator factor eIF-2α, resulting in a cessation of protein synthesis. In addition, PKR is implicated in inhibiting VSV replication in mice (2). However, PLSCR1 did not affect IFN-induced levels of PKR. Expression of PLSCR1 was also associated with modestly enhanced expression of IRF7, which could potentially lead to IFN synthesis, thus further amplifying the antiviral response (Fig. 3) (27). The apparent enhancing effect of PLSCR1 on any particular gene was in the range of a 1.5- to >5-fold, with the combined effect on presumably several ISGs resulting in a significant negative impact on virus replication.

Effect of PLSCR1 on VSV replication. Although PLSCR1 appeared to enhance the expression of a number of genes, a direct effect of this protein on virus replication is also possible. Therefore, to determine how PLSCR1 was affecting VSV replication, we analyzed different stages in the virus cycle. The location of PLSCR1 in the cell membrane suggested a possible effect on virus adsorption and/or uptake. However, these steps in the virus replication cycles were unaffected by PLSCR1. In contrast, there was a substantial increase in primary (N) transcript accumulation in PLSCR1$^{-/-}$ (KO2) cells. Reduction in VSV primary transcript accumulation by IFN in either the absence or presence of PLSCR1 was substantial. The effect of PLSCR1 on VSV replication is superimposed on a larger IFN antiviral effect. As a result, we were unable to accurately determine whether the reduction by IFN in the two cell types was comparable. Our findings are consistent with a previous report demonstrating that IFN treatment affects VSV replication at the level of primary transcription (5). However, different studies localized the effect of IFN against VSV to other stages in the replication cycle, including protein synthesis (34) and virus assembly (22). Recently, it was demonstrated that IFN inhibits VSV entry into human epithelial cells by producing soluble secreted antiviral factors (S. Bose and A. K. Banerjee, unpublished data). Therefore, there are clearly cell-type-specific differences in the anti-VSV mechanism of IFNs. The present study does not rule out an effect on viral protein synthesis because it is difficult to measure an effect on protein synthesis when there is potent inhibition of viral primary transcription. IFN was able to reduce VSV primary transcript accumulation even in the PLSCR1$^{-/-}$ (KO2) cells, perhaps because the ISG(s) responsible for this effect was still induced, albeit to a lower extent than in the PLSCR1-positive KI cells (Fig. 7). Although expression of PLSCR1 was associated with modestly enhanced IFN-induction of OAS2 (encoding a 2-5A synthetase), there were no RNase L-mediated rRNA cleavage products in IFN-treated, VSV-infected KI cells (data not shown). Therefore, RNase L action against viral RNA is unlikely to be responsible for the decreased accumulation of VSV primary transcripts observed in the PLSCR1$^{-/-}$ cells (data not shown). The effect of PLSCR1 on a budding mutant of VSV was also investigated. The N-terminal, cytoplasmic domain of mPLSCR1 and hPLSCR1 contains PPXY motifs typical of WW-binding domains that could potentially interfere with virus budding. These motifs are similar to the PY motif or late-budding domain of the VSV M protein (PPPY) and of other members of the *Rhabdoviridae*, *Retroviridae*, and *Filoviridae* (18). VSV yields were compared from KO2 and KI cells infected with wild-type and mutant VSV in which the PPPY budding domain of the M protein was altered to AAPA to impair viral release (17, 23). However, yields of both wild-type and mutant VSV were similarly decreased in the KI cells compared with the KO2 cells (Fig. 9). These data suggest that, irrespective of any potential antiviral effect of PLSCR1 at the stage of virus assembly and budding from the plasma membrane, PLSCR1 must also exert an inhibitory or antiviral action prior to this terminal event in viral replication.

It was apparent from these studies that the observed antiviral effect of PLSCR1 extended beyond VSV. Replication of both VSV and EMCV were suppressed by expression of PLSCR1 in the human Hey1B cell line. In PLSCR1$^{-/-}$ MEFs, an antiviral effect of ectopically expressed PLSCR1 was observed against both VSV and the murine retrovirus Moloney murine leukemia virus (Fig. 5 and data not shown). While our results suggest that the broad antiviral effect mediated by PLSCR1 is related to enhanced expression of certain antiviral genes, the specific ISGs responsible for the inhibition of VSV, EMCV, and Moloney murine leukemia virus replication observed in this study are unknown. However, our findings indicate that PLSCR1 is an amplifying factor in the expression of certain critical antiviral genes that collectively have a large impact on virus growth. Furthermore, our gene array results provide a relatively short list of interesting candidate genes, some of which are responsible for potent inhibition of viral replication (Fig. 3). Exploring the specific functions of these genes targeted by PLSCR1 will be a new direction for investigating how IFNs protect cells against viral infections.

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