Palmitoylation of Phospholipid Scramblase 1 Controls Its Distribution between Nucleus and Plasma Membrane†

Therese Wiedmer, Ji Zhao, Meera Nanjundan, and Peter J. Sims*

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

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ABSTRACT: Phospholipid scramblase 1 (PLSCR1) is a Ca^{2+}-binding, endofacial plasma membrane protein thought to contribute to the transbilayer movement of phosphatidylserine and other membrane phospholipids that is observed upon influx of calcium into the cytosol. Expression of PLSCR1 is markedly induced by interferon and other cytokines, and PLSCR1−/− bone marrow cells exhibit defective myeloid proliferation and differentiation in response to stimulation by select growth factors, implying that PLSCR1 also functions in cytokine signaling or response pathways. PLSCR1 is multiply palmitoylated and partitions into membrane lipid raft domains. We have now identified the Cys-rich sequence ^{184}CCCPCC^{189} in PLSCR1 as required for palmitoylation of the polypeptide. Mutation of these five cysteines abrogates PLSCR1 trafficking to the plasma membrane and results in virtually all of the expressed protein localizing to the nucleus. Consistent with this observation, cell treatment with the palmitoylation inhibitor, 2-bromo-palmitate, results in a marked redistribution of endogenous PLSCR1 from plasma membrane to nucleus. In a small percentage of untreated cells, predominantly nuclear localization of PLSCR1 is also observed. Furthermore, PLSCR1 is also found in the nucleus following its cytokine-induced expression. These data suggest that under the circumstance of rapid biosynthesis in response to gene induction by cytokines, PLSCR1 traffics into the nucleus, implying a potential nuclear function for this protein.

Phospholipid scramblase 1 (PLSCR1)[1] is a member of a recently identified family of membrane proteins that has been proposed to contribute to the reorganization of plasma membrane phospholipids (PLs) in activated platelets and in injured or apoptotic cells exposed to elevated intracellular Ca^{2+} (*1*−*4*). In addition to a putative role in promoting transbilayer redistribution of plasma membrane PLs through this Ca^{2+}-activated PL scramblase pathway, PLSCR1 has also been reported to be a substrate of several kinases that participate in cell proliferative, differentiation, or apoptotic responses, including protein kinase C*δ*, c-Abl, and tyrosine kinase(s) coupled to both IgE and epidermal growth factor (EGF) receptors (*5*−*8*). PLSCR1 is a palmitoylated protein that partitions with flotillin and EGF-receptors into caveolar-like membrane raft domains (*8*, *9*).

Although suggested to play a role in the redistribution of plasma membrane PLs through the PL scramblase pathway, the actual cellular function(s) of PLSCR1 and related members of this gene family remains largely unresolved. The putative role of PLSCR1 in mediating Ca^{2+}-dependent accelerated transbilayer migration of plasma membrane PLs derived from its capacity to mediate this function in reconstituted membrane systems and the apparent correlation between levels of endogenous expression of cellular PLSCR1 and the propensity of various cells to expose phosphatidylserine in response to influx of Ca^{2+} (*1*, *2*, *10*). Subsequently, it was reported that the Thr-phosphorylation of PLSCR1 by cellular protein kinase C*δ* served to promote surface exposure of plasma membrane phosphatidylserine in Jurkat cells induced to apoptosis, implicating a role for protein phosphorylation in activation of PLSCR1's PL-scrambling function (*5*). Nevertheless, elevation of PLSCR1 expression by transfection was variably found to increase plasma membrane PL scramblase activity (*5*, *10*, *11*), whereas no increase in such plasma membrane PL scramblase activity was detected upon interferon-induced expression of PLSCR1 (*12*) or following Tyr-phosphorylation of PLSCR1 by activated c-Abl kinase (*7*).

In yeast, there is evidence that the single apparent PLSCR orthologue (YJR100C) is a stress-induced gene (*13*), whereas human and mouse PLSCR1 expression is highly induced by interferons (*12*, *14*) and by various other cytokines including stem cell factor (SCF; c-kit ligand) (*15*), granulocyte colony stimulating factor (G-CSF) (*15*), and EGF (*8*). Mice with targeted deletion of the PLSCR1 gene show delayed fetal and perinatal granulocyte production and a defect in the proliferation and differentiation of myeloid cells in response to stimulation by SCF or G-CSF (*15*). Taken together, these data suggest that PLSCR1 participates in immune/stress responses, cell cycle regulation, or apoptosis, potentially by regulating the metabolic or mitotic responses elicited through multiple cytokine receptors.

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* To whom correspondence should be addressed. Tel.: (858) 784-2307. Fax: (858) 784-2777. E-mail: psims@scripps.edu.

[1] Abbreviations: PL, phospholipid; PLSCR, phospholipid scramblase; EGF, epidermal growth factor; G-CSF, granulocyte colony stimulating factor; SCF, stem cell factor or c-Kit ligand; mab, monoclonal antibody; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis.

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To better understand how the posttranslational modifications of PLSCR1 might relate to its biologic function, we have examined the contribution of thiolesterification. We found that in the absence of palmitoylation, virtually all of the expressed PLSCR1 localizes to the nucleus. Whereas under normal growth conditions nuclear PLSCR1 is detected in only a small percentage of cells, under cytokine stimulation there is a marked increase in the number of cells with PLSCR1 located in the nucleus.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were from New England BioLabs (Beverly, MA) and pcDNA3 vector was from Invitrogen (Carlsbad, CA). Fetal bovine serum, goat serum, and normal mouse IgG were from Sigma. DMEM cell culture medium was from Mediatech (Herndon, VA), Lipofectamine PLUS transfection reagent from Gibco Invitrogen Corp. (Grand Island, NY), and propidium iodide from Molecular Probes (Eugene, OR). Protein G Sepharose and Fluorographic Amplify were from Amersham (Piscataway, NJ). ^{3}H-palmitate was from NEN Life Science Products, Inc. (Boston, MA), 2-bromopalmitate from Aldrich (Milwaukee, WI), and universal interferon was from PBL Biomedical Laboratories (New Brunswick, NJ). Protease inhibitor cocktail tablets (containing inhibitors of serine and cysteine proteases) were from Roche Molecular Biochemicals (Indianapolis, IN), RNAse was from Quiagen (Valenica, CA), and SuperSignal chemiluminescence kit was from Pierce (Rockford, IL). Murine monoclonal antibody (mab) 4D2 specific for human PLSCR1 was raised in our laboratory (*12*). Rabbit antibody against histone was from Santa Cruz, Inc. (Santa Cruz, CA). FITC conjugated goat anti mouse and anti-rabbit IgGs were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture. Mouse fibroblast cell line SVT2 and human fibrosarcoma HT1080 were cultured in DMEM containing 10% fetal bovine serum, and the human ovarian carcinoma cell line Hey1B was cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Mutagenesis. Human PLSCR1 cDNA (1445bp, GenBank accession number AF008445) was cloned into pcDNA3 vector. In addition to individual mutations of the 18 Cys residues in human PLSCR1, the following Cys to Ala mutations within the segment ^{184}CCCPCC^{189} were constructed by oligonucleotide-directed mutagenesis as previously described (*16*): (a) ^{184}AAAPAA^{189}; (b) ^{184}AAAPCC^{189}; and (c) ^{184}CCCPAA^{189}.

Metabolic Labeling of WT and Cys-Mutant PLSCR1 with [^{3}H]-Palmitate. Wild type (WT) and mutant constructs of human PLSCR1 were transfected into SVT2 cells by electroporation. After 24 h of culture, the cells were incubated at 37 °C overnight with 400 μCi/mL [^{3}H]-palmitate (specific activity 60 Ci/mmol) in DMEM containing 20% fetal bovine serum (dialyzed against PBS, pH 7.5) and 5 mM sodium pyruvate. Cells were harvested and washed, and membrane proteins were extracted into a lysis buffer containing 1% Triton X-100, 5 mM EDTA, and protease inhibitor cocktail in PBS, pH 7.4.

Immunoprecipitation and SDS−PAGE. Insoluble material was removed from cell extracts by centrifugation, and the supernatants were precleared by incubation for 30 min at room temperature with 50 μg of normal mouse IgG and 10 μL of protein G Sepharose per 500 μL of lysate. PLSCR1 was precipitated by incubation at room temperature for 3 h with 25 μg of mab 4D2 mab and 10 μL of protein G Sepharose per 0.5 mL of cell lysate. The beads were washed exhaustively, and bound protein was eluted into 5% SDS−PAGE sample buffer containing 1 mM DTT by incubation for 3 min at 70 °C. After addition of 2 mM iodoacetic acid, each sample was subjected to polyacrylamide gel electrophoresis (12% Tris-glycine Novex gels). In certain experiments, samples were subjected to deacylation by incubation for 1 h in 1 M Tris-hydroxylamine-HCl, pH 7.2, prior to SDS−PAGE. Identical samples incubated in 1 M Tris-HCl, pH 7.2, served as controls (*9*).

Fluorography. The SDS−PAGE gels were fixed in 50% ethanol and 5% acetic acid for 1 h. After washing for 1 h in 20% ethanol and 5% acetic acid followed by three washes with distilled water, the gels were soaked in Fluorographic Amplify for 30 min, dried, and exposed to preflashed Kodak XAR-5 film at −80 °C.

Western Blotting. Proteins were resolved on 12% Tris-glycine Novex gels and electrophoretically transferred to nitrocellulose membranes. After blocking with 5% milk, the membranes were incubated for 1 h with primary antibody and 1 h with appropriate horseradish peroxidase-conjugated secondary antibody. Blots were developed using SuperSignal chemiluminescence substrates.

Fluorescence Microscopy. Cells grown on glass coverslips were washed in PBS and fixed with 4% formaldehyde in PBS for 30 min. After permeabilization by 0.2% Triton X-100 in PBS for 5 min, cells were incubated with 0.1 mg/mL RNAse in 2% whole goat serum/PBS for 30 min at 37 °C, followed by incubation with mab 4D2, 5 μg/mL in 2% goat serum/PBS for 1 h. Cells were stained with FITC-labeled goat anti-mouse IgG, 4 μg/mL in PBS for 1 h, followed by nuclear counterstain with propidium iodide 0.1 μg/mL in PBS for 10 min. Coverslips were mounted on glass slides, and samples were analyzed on a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a Zeiss X60 objective. Images were collected using Bio-Rad's LaserSharp software. Specificity of staining observed for 4D2 was evaluated by cell staining with the identical concentration of an isotype-matched antibody.

RESULTS

Identity of Cys Residues in PLSCR1 Required for Protein Palmitoylation. To identify the cysteine residues in PLSCR1 that are required for thiolesterification, we undertook systematic mutagenesis of the 18 Cys residues contained within the polypeptide and measured metabolic incorporation of ^{3}H-palmitate following cell transfection with these various mutant constructs. Palmitoylation of the polypeptide (as deduced by covalently bound ^{3}H-palmitate) was observed in all cases in which Ala was substituted either for each single Cys residue, or, for all tandem Cys residues within adjacent Cys repeats (Figure 1 , data not shown). By contrast, we observed complete loss of palmitoylation of the polypeptide when all five Cys residues of the Cys-rich segment ^{184}CCCPCC^{189} were mutated, suggesting that these residues are the sites of palmitoylation within the PLSCR1 polypeptide (Figure 1). As also illustrated by these data, complete

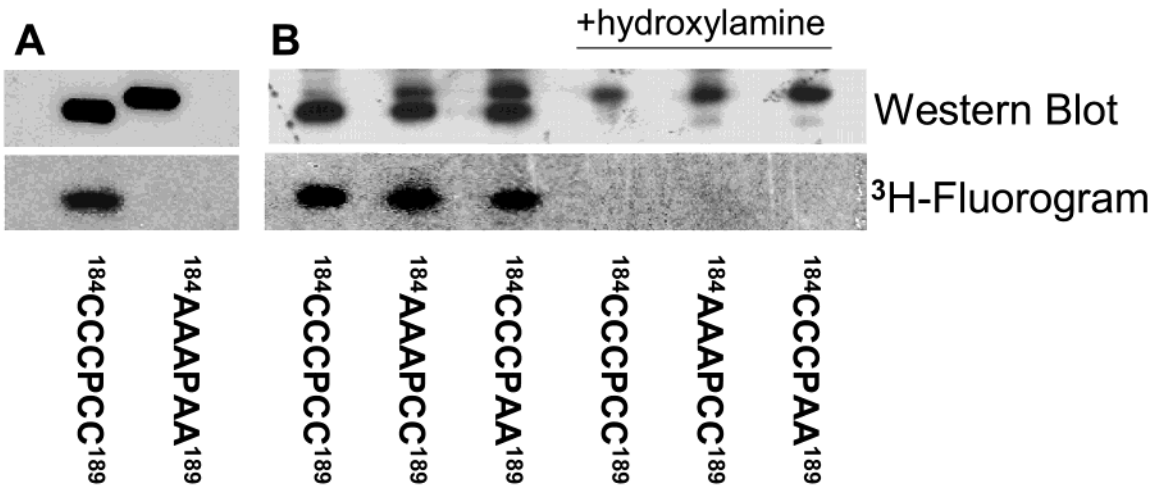

FIGURE 1: Mutagenesis reveals a cluster of five cysteines as sites of palmitoylation of PLSCR1. Figure depicts mapping of Cys residues contained between residues 184−189 in human PLSCR1 as the sites required for protein palmitoylation. PLSCR1 constructs (WT or Ala substituted at Cys 184, 185, 186, 188, and 189) in pcDNA3 were transiently expressed in murine SVT2 cells. After culture with ^{3}H-palmitate, the cells were lysed in TX-100, PLSCR1 was immunoprecipitated with mab 4D2 (specific for human PLSCR1), and Western blotting and fluorography were performed (A). In panel B, WT and the Cys-mutant PLSCR1 constructs indicated were treated without or with 1 M hydroxylamine before Western blotting and fluorography were performed. Note that total elimination of incorporated ^{3}H-palmitate required mutation at all 5 Cys, whereas some incorporation of ^{3}H-palmitate into PLSCR1 was still observed after mutagenesis of either Cys 184−186 or Cys 188−189 (panel B). Elimination of the thiolesterified fatty acid was accompanied by ∼5 kDa increase in the apparent molecular weight of the polypeptide by SDS−PAGE.

loss of thiolesterified fatty acids, either by Ala substitution at all five Cys residues spanning ^{184}Cys−^{189}Cys or by hydrolysis of WT PLSCR1 with hydroxylamine, resulted in a distinct decrease in mobility of the polypeptide by SDS−PAGE. When less than five of these Cys residues in PLSCR1 were mutated, a doublet band was observed, with most of the protein comigrating with the WT protein, and a smaller component comigrating with the slower deacylated polypeptide (Figure 1, data not shown). As ^{3}H-palmitate was detected only in those protein bands comigrating with WT PLSCR1, these data suggest that the apparent mobility shift is observed only when palmitoylation is completely eliminated.

Mutation at Sites of Palmitoylation Results in Trafficking to the Nucleus. PLSCR1 is normally detected in the plasma membrane and various internal membrane pools (*8*, *10*). By contrast, when we expressed mutant PLSCR1 that cannot be palmitoylated, a considerable portion of the polypeptide was found diffusely distributed within the nucleus, with little to none of the protein at the cell surface (Figures 2 and 3). The nuclear localization of PLSCR1 mutated to prevent its palmitoylation was consistently found in multiple independent transfections and was observed both during transient expression and after selection of stably transformed cell lines expressing various levels of the mutant polypeptide (data not shown).

Treatment with 2-Bromo-palmitate Results in Nuclear Expression of Endogenous PLSCR1 Antigen. To determine whether endogenous wild-type PLSCR1 would also traffic to the nucleus under conditions in which metabolic palmitoylation was prevented, expression of PLSCR1 in HT1080 cells was induced with α-interferon during culture in the presence of 2-bromo-palmitate, a competitive inhibitor of the transfer of palmitoyl-CoA to the cysteinyl thiol (*12*, *17*). As was observed for transfected mutant ^{184}AAAPAA^{189} PLSCR1, a considerable amount of the expressed WT (^{184}CCCPCC^{189}) PLSCR1 antigen was also found to be distributed within the nucleus when cells were grown in 2-bromo-palmitate (Figure 4).

Cytokine Induction of Nascent PLSCR1 Can Also Result in Trafficking to the Nucleus. PLSCR1 expression is known to be transcriptionally up-regulated by the interferons (*12*, *14*) and by several growth factors, including EGF (*8*), SCF (*15*), and G-CSF (*15*). As illustrated by the data of Figure 5, in certain cell lines we observed a distinct nuclear localization of the newly expressed PLSCR1 antigen following cytokine induction of protein synthesis, even when a cell culture was performed in the absence of 2-bromo-palmitate or other inhibitor of palmitoylation. The number of cells showing prominent nuclear localization of PLSCR1 after cytokine induction of protein synthesis was found to be quite variable, dependent upon both the time after exposure to cytokine and the particular cell line utilized for cytokine stimulation. Among various cell lines tested, the human ovarian tumor cell line HEY1B was found to be particularly prone to exhibit nuclear localization of PLSCR1, whereas in HT1080 such nuclear localization was rarely observed unless palmitoylation was inhibited with 2-bromo-palmitate (cf. Figure 4).

DISCUSSION

Our data indicate that (1) trafficking of PLSCR1 to the plasma membrane is dependent upon palmitoylation of the polypeptide; (2) a Cys-rich string (^{184}CCCPCC^{189}) that is distal from the predicted transmembrane domain of the polypeptide is required for protein palmitoylation; (3) in the absence of palmitoylation, PLSCR1 traffics into the nucleus; and (4) whereas under basal conditions, virtually all of the expressed PLSCR1 antigen is in extranuclear locations (plasma membrane and endosomal pools), prominent nuclear expression of the protein can be observed following cytokine-induced transcriptional up-regulation.

It is generally accepted that palmitoylation plays a major function in targeting proteins to specialized cholesterol and

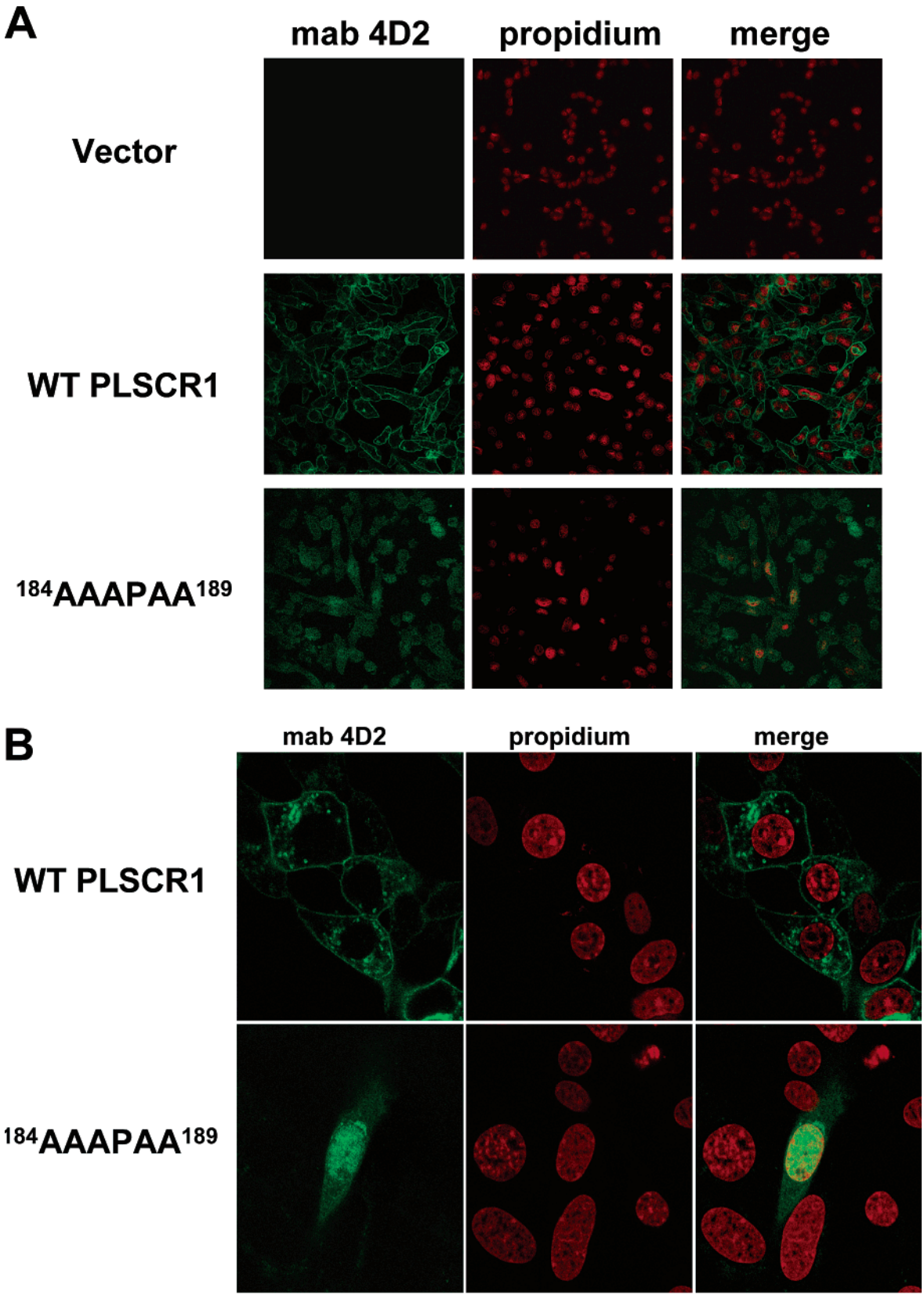

FIGURE 2: PLSCR1 lacking sites of palmitoylation fails to traffic to plasma membrane and is expressed in the nucleus. (A) Confocal fluorescence images of murine SVT2 fibroblasts stably transfected with pcDNA3 alone (vector) or pcDNA3 containing cDNA for WT human PLSCR1 or the ^{184}AAAPAA^{189} mutant. Expressed human PLSCR1 antigen was detected with mab 4D2 (specific for human PLSCR1) and FITC-conjugated goat anti-mouse IgG. Figure shows a typical field of cells examined at 400× magnification. Note contrast between prominent plasma membrane staining in WT vs cytoplasmic and nuclear staining observed for cells expressing the ^{184}AAAPAA^{189} mutant (for several cells, the plane of focus is either above or below the nucleus). (B) Confocal fluorescence images at 1000× magnification of SVT2 cells transfected with either WT PLSCR1 or the ^{184}AAAPAA^{189} mutant. Images shown are typical of more than 20 fields examined after multiple independent transfections. No green fluorescence was detected when isotype matched control IgG was substituted for 4D2 (not shown).

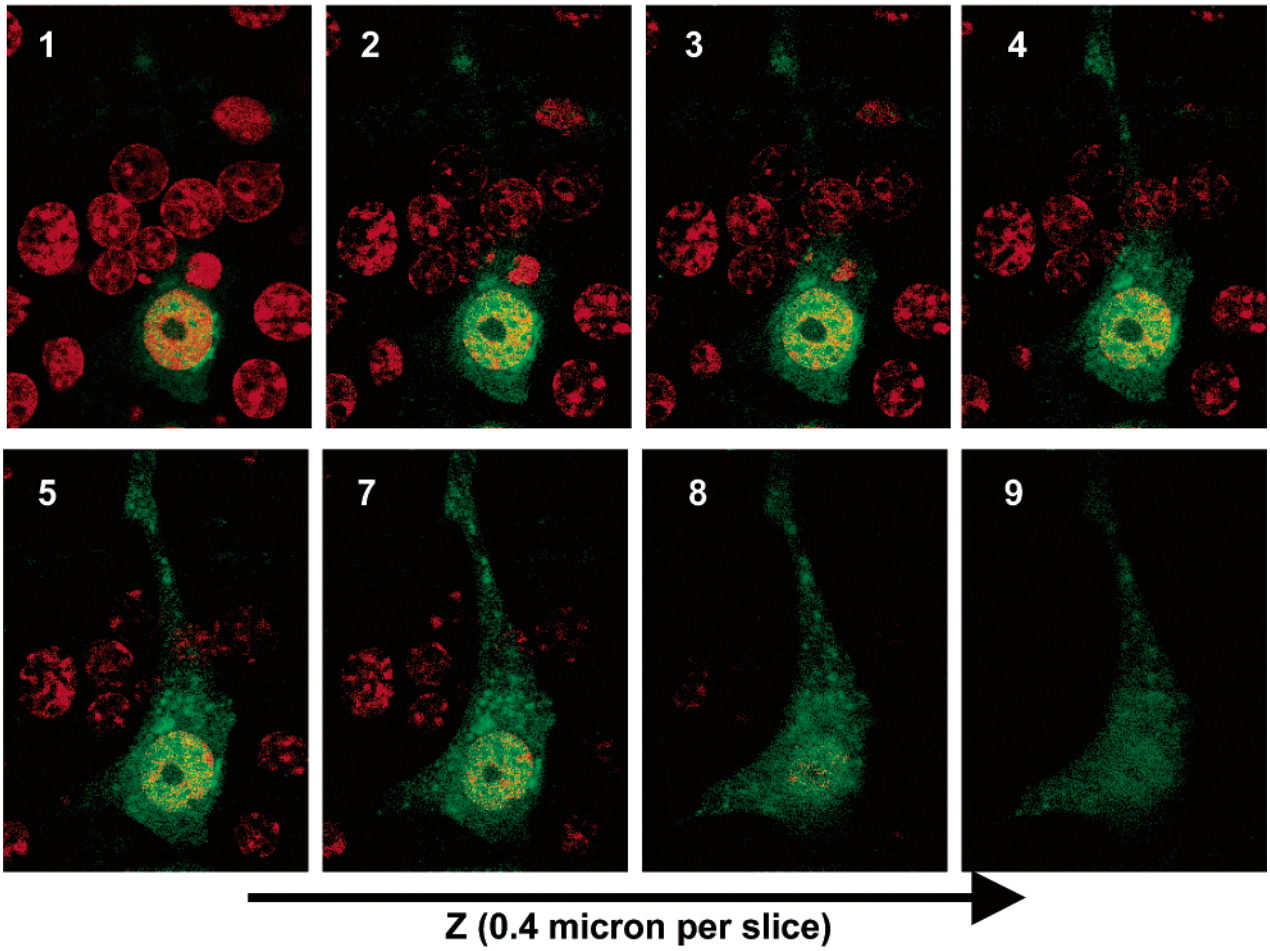

FIGURE 3: Confocal analysis confirms nuclear localization of PLSCR1 lacking palmitoylation sites. Figure shows successive 0.4 μm Z-plane steps through a single cell expressing the PLSCR1 ^{184}AAAPAA^{189} mutant to confirm intranuclear distribution of the polypeptide. Green channel is FITC-mab 4D2; red channel is propidium iodide.

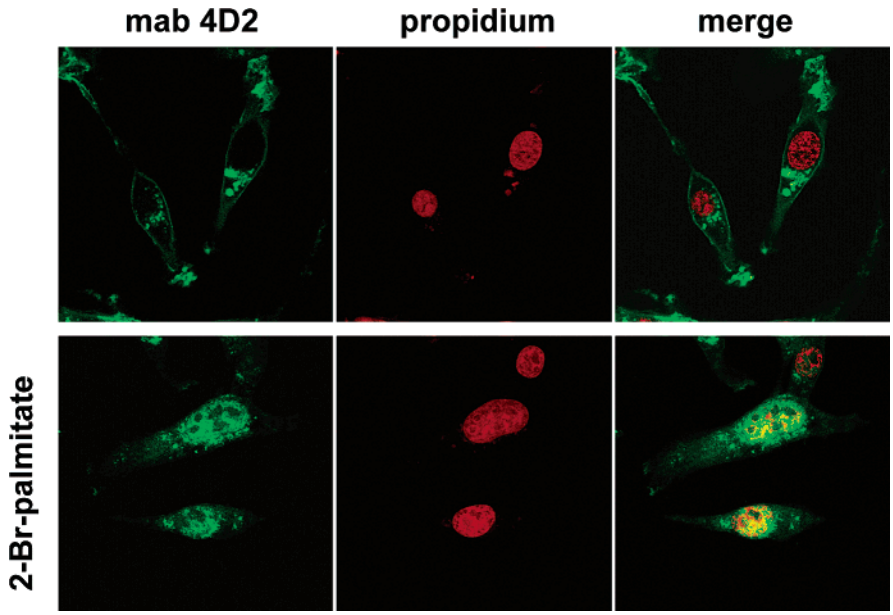

FIGURE 4: Inhibition of palmitoylation results in nuclear localization of PLSCR1. Human HT1080 cells were cultured overnight in 1000 U/mL interferon and either vehicle (upper panels) or 2-bromo-palmitate (lower panels), and the endogenous PLSCR1 antigen was detected with mab 4D2 (green channel). Nuclear DNA was stained with propidium iodide (red channel).

sphingomyelin-enriched plasma membrane domains, or lipid rafts (*18*). These domains are particularly enriched in many signaling molecules and thus are considered to be specialized sites for assembly of growth factor receptor signaling complexes and other membrane receptor complexes, as well as to play a role in endocytic trafficking of these receptors

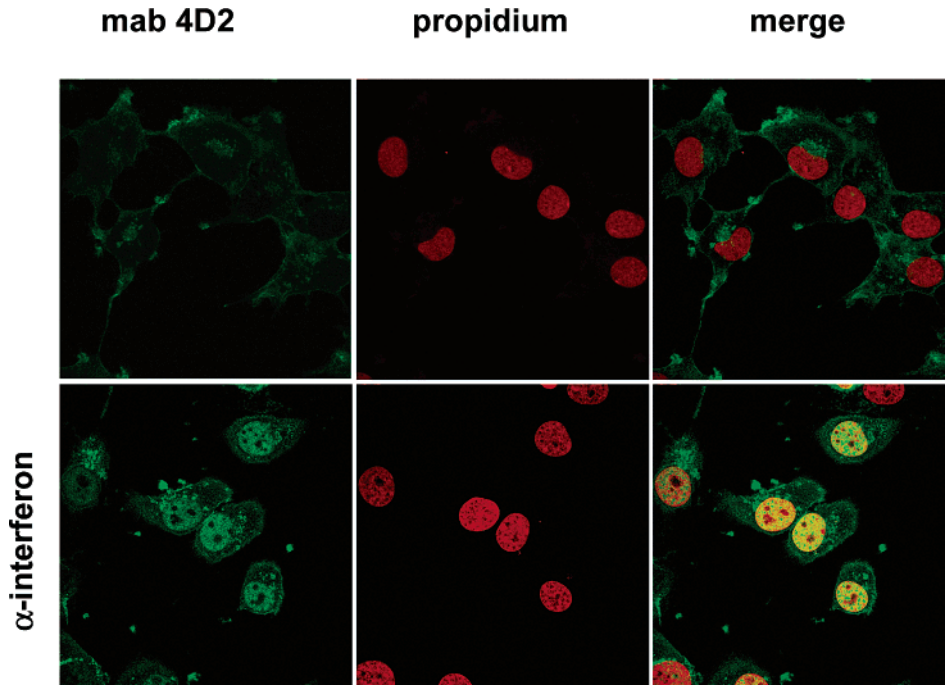

FIGURE 5: Nuclear localization of PLSCR1 after cytokine induction. Confocal immunofluorescence of human HEY1B cells following treatment (12 h) with 1000 U/mL interferon to induce PLSCR1 expression. PLSCR1 antigen was detected with mab 4D2 (green channel). Nuclear DNA was stained with propidium iodide (red channel). The extent of nuclear trafficking of PLSCR1 observed after cytokine induction varied considerably with cell type and was most prominent for the HEY1B cell line.

from the plasma membrane (*19*, *20*). We recently showed that PLSCR1 co-localizes with EGF receptors in lipid rafts and that PLSCR1 is both phosphorylated and binds to the adapter protein Shc upon activation of this receptor, implying a potential role for PLSCR1 in signaling through EGF receptors and potentially related growth factor receptor complexes (*8*). Cell stimulation by EGF was also found to initiate endocytosis of plasma membrane PLSCR1 concomitant with that of the activated EGF receptors. Whereas most of the internalized EGF receptors traffic into proteosomes for degradation, the internalized PLSCR1 was not degraded but found to recycle to the plasma membrane (*8*). It remains to be determined whether a dynamic palmitoylation (or depalmitoylation) of membrane-associated PLSCR1 regulates its localization in and out of lipid raft domains or subsequent trafficking between endosomal and plasma membrane pools.

As noted, our data indicated that the Cys-rich string ^{184}CCCPCC^{189} is required for protein palmitoylation and suggest that these residues are the actual sites of palmitoylation. This location of thiolesterified Cys residues within the PLSCR1 polypeptide is unusual. Proteins that are cysteine palmitoylated but are not also covalently modified by either isoprenylation or myristoylation generally fall into two categories: type I membrane proteins are typically palmitoylated on cysteines located adjacent to or just within a transmembrane domain, whereas type III proteins are palmitoylated near N- or C-termini (*19*). By contrast, PLSCR1 does not appear to fit either of these classifications, as the palmitoylated residues are located within a central segment of the polypeptide that is separated by approximately 100 amino acids from the only predicted transmembrane domain (residues 291−309). Of interest, the ^{184}CCCPCC^{189} motif in PLSCR1 is quite similar to that recently identified as the palmitoylation site in phosphatidylinositol 4-kinase type IIb, (residues ^{173}CCPCC^{177}), a protein without an obvious transmembrane domain (*21*). It will be of interest to determine whether similar Cys-rich clusters identified in other proteins will also be found to be potential sites for palmitoylation.

A unique finding of the present study is the observation that PLSCR1 traffics into the nucleus in those circumstances where palmitoylation of the polypeptide does not occur. Nuclear localization was observed both for mutant PLSCR1 in which Cys residues within the palmitoylation motif ^{184}CCCPCC^{189} were mutated to Ala, as well as in the circumstance when palmitoylation was metabolically inhibited by 2-bromo-palmitate, a competitive inhibitor of the transfer of palmitoyl-CoA to the cysteinyl thiol. PLSCR1 was also detected within the nucleus following its cytokine-induced expression, which may reflect nuclear import of the nascent polypeptide prior to its complete posttranslational thiolesterification. It is of note that multiple cytokines, including type I and II interferons (*12*, *14*), SCF (c-kit ligand) (*15*), G-CSF (*15*), EGF (*8*), as well as related peptide growth factors (Nanjundan, M., Sun, J., Wiedmer, T., and Sims, P. J., unpublished data) have all been found to markedly increase cellular expression of PLSCR1, a circumstance where we observed nuclear distribution of the newly expressed polypeptide. It is therefore tempting to speculate that (i) nuclear import of this polypeptide contributes to some common late posttranscriptional event(s) that is initiated by these diverse cytokines; and (ii) the defect in cytokine-stimulated proliferation and maturation that is observed in PLSCR1−/− hematopoietic precursor cells (*15*) relates to an activity of PLSCR1 within the nucleus that contributes to normal cell growth and maturation.

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