Phospholipid Scramblase 1 Is Imported into the Nucleus by a Receptor-Mediated Pathway and Interacts with DNA[†,‡]

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ABSTRACT: Phospholipid scramblase 1 (PLSCR1) is a multiply palmitoylated, Ca^{2+}-binding, endofacial plasma membrane protein originally identified by its capacity to accelerate transbilayer movement of membrane phospholipids. We recently reported that when palmitoylation of PLSCR1 does not occur, it is localized to the nucleus rather than the plasma membrane. Nuclear localization of PLSCR1 was also observed upon induction of its *de novo* synthesis by cytokines such as interferon α that activate the *PLSCR1* gene. Despite its capacity to enter the nucleus, its sequence does not predict a nuclear localization signal. To gain insight into the mechanism and potential significance of nuclear PLSCR1, we investigated the conditions required for its import and retention in the nucleus. We show that nuclear localization of PLSCR1 is dependent on cytosolic factors and energy. Furthermore, we show that PLSCR1 is specifically transported into the nucleus by the importin α/β import pathway, and binds directly and with high affinity to importin α. Analysis of deletion mutants suggested that the NLS of PLSCR1 is between residues 242 and 290 and, furthermore, that a peptide within this region encompassing residues ^{257}GKISKHWTGI^{266} is sufficient for nuclear import when conjugated to BSA. In addition, in intact cells, mutation of positively charged amino acids within this putative NLS in the full-length protein completely blocked its entry into the nucleus, consistent with its role in targeting PLSCR1 to the nucleus. Release of PLSCR1 from the nucleus was only observed after treatment of cells with both detergent and an elevated NaCl concentration, or following DNase treatment of the nucleus, suggesting ionic interactions of PLSCR1 with a nuclear component bound to genomic DNA or directly with genomic DNA. Purified PLSCR1 was also found to bind directly to a genomic DNA−cellulose conjugate, and its elution from DNA also required an elevated NaCl concentration. These data support a mechanism of receptor-mediated nuclear import of PLSCR1 and suggest a potential nuclear function for this plasma membrane protein.

Phospholipid scramblase 1 (PLSCR1)[1] is a multiply palmitoylated, calcium-binding, endofacial plasma membrane protein that has been proposed to contribute to accelerated transbilayer movement of membrane phospholipids at elevated calcium concentrations (*1*−*3*). Despite its suggested role in the redistribution of plasma membrane phospholipids (PLs) through the PL scramblase pathway, the actual cellular function(s) of PLSCR1 and related members of this gene family remains largely unresolved.

PLSCR1 has been reported to undergo phosphorylation by kinases that participate in cell proliferation, maturation, and apoptotic responses, including c-Abl, c-Src, and protein kinase Cδ, and tyrosine phosphorylation of PLSCR1 has been observed to occur in response to epidermal growth factor and other growth factors (*4*−*7*). Deletion of PLSCR1 was found to retard proliferation and maturation of certain hematopoietic stem cells under growth factor stimulation, and was also shown to attenuate the activation of c-Src, a kinase known to play a central role in receptor signaling (*7*, *8*). The level of PLSCR1 expression appears to increase with cell maturation, and this gene has been shown to be transcriptionally activated by the interferons as well as by multiple growth factors (*8*, *9*).

In addition to its apparent activities at the plasma membrane, it was recently reported that PLSCR1 has the potential to enter the nucleus. In particular, nuclear trafficking of PLSCR1 was observed in circumstances where thiol esterification of the polypeptide was prevented, either by mutation at its sites for palmitoylation or by cell treatment with 2-bromopalmitate, a competitive inhibitor of palmitoyl-CoA (*10*). In the absence of inhibitors of palmitoylation, a substantial nuclear component of PLSCR1 was also observed

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[1] Abbreviations: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MBP, maltose binding protein; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; NTF2, nuclear transport factor 2; pAb, polyclonal antibody; PL, phospholipid; PLSCR1, phospholipid scramblase 1; WGA, wheat germ agglutinin.

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in certain cells when the synthesis of this protein was transcriptionally induced by treatment with interferon α, suggesting that the nuclear-localized PLSCR1 might play some role in the overall cellular response to those cytokines and growth factors that are known to induce expression of the PLSCR1 gene.

Bidirectional nuclear transport of macromolecules takes place through large protein channels termed the nuclear pore complexes (NPC) that are embedded in the nuclear membrane (*11*). Ions, metabolites, and small proteins (less than ~40 kDa) move through the NPC by passive diffusion, but the majority of larger molecules are transported by signal- and energy-dependent mechanisms. Receptor-mediated nucleocytoplasmic transport allows for trafficking of nuclear proteins involved in transcription, DNA replication, and chromatin remodeling as well as of mRNAs, tRNAs, and rRNAs that are transcribed in the nucleus but function in the cytoplasm (*12*).

The amino acid sequence of PLSCR1 does not contain a classical nuclear localization signal (NLS) motif. Furthermore, its relatively small size (37 kDa) raises the possibility that the observed nuclear distribution was simply the result of passive diffusion through the NPC. We were therefore interested in addressing the question of whether PLSCR1 is imported into the nucleus by a specific receptor-mediated import mechanism. We expressed the MBP−PLSCR1 protein (MBP−PLSCR1) as a recombinant protein and used a permeabilized cell assay to study the requirements for nuclear import. Using this assay, we show that the nuclear import of PLSCR1 requires cytosol and energy and is inhibited by low temperatures and wheat germ agglutinin (WGA), an inhibitor of signal-dependent nuclear transport. In addition, we show that PLSCR1 is imported into the nucleus by an importin α/*β*- and Ran-dependent mechanism, and that the interaction with importin α is direct with a nanomolar apparent affinity. Deletional mutation analysis of PLSCR1 suggested that nuclear localization of PLSCR1 involved a sequence contained between amino acid residues 257 and 266, close to the C-terminus, and mutation of positively charged amino acids within this region blocked nuclear import of full-length PLSCR1 in intact cells. Interestingly, once imported into the nucleus, PLSCR1 did not diffuse out with detergent solubilization of the nuclear envelope, and only sequential treatment with both Triton X-100 and NaCl, or treatment with both Triton X-100 and DNase, caused the release of previously imported PLSCR1 from the nucleus. In addition, we observed PLSCR1, an acidic protein, to bind specifically to a genomic DNA−cellulose conjugate and to elute from DNA at elevated salt concentrations similar to those required to extract it from the cell nucleus. These observations suggest that nuclear import of PLSCR1 is an active process mediated by the importin α/*β* pathway through a direct interaction with an NLS expressed by the PLSCR1 polypeptide. These data also raise the possibility of a previously unrecognized nuclear function for this protein, mediated through a potential interaction with genomic DNA.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Deletion mutants of MBP−PLSCR1 were generated using PCR with appropriate oligonucleotide primers, and the PCR-derived DNAs were cloned into the pMal-C2 vector as *Eco*RI and *Sal*I fragments. Recombinant nuclear import factors were derived as described in detail in refs *13* and *14*. Human PLSCR1 cDNA or mutant PLSCR1 (^{184}AAAPAA^{189}) in the pcDNA3 vector was constructed as previously described (*10*). The mutant PLSCR1(^{184}AAAPAA^{189,257}GAISAAWTGI^{266}) was constructed by oligonucleotide-directed mutagenesis with appropriate oligonucleotide primers, and the PCR-derived DNA was cloned into the pcDNA3 vector as *Eco*RI and *Bam*HI fragments. The sequences of the constructs were verified by DNA sequencing.

Expression and Purification of Recombinant Proteins. MBP−PLSCR1 and deletion mutants were expressed in *Escherichia coli* BL21 cells or TB1 cells and purified on amylose resin as previously described (*3*). These proteins were dialyzed into transport buffer [20 mM Hepes (pH 7.4), 110 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT] before being used in the various experiments. Expression and purification of His-importin α, importin *β*, mutant His-importin *β* (deleting the Ran binding site), Ran, the RanQ69L mutant, nuclear transport factor 2 (NTF2), and GST-M9 (a cargo of the nuclear import receptor, transportin) were performed as previously described (*13*, *14*).

Nuclear Import Assay. For analysis of nuclear import in digitonin-permeabilized adherent NRK cells, nuclear import *in vitro* assays, substrate visualization, and preparation of HeLa cytosol (by digitonin lysis) were carried out essentially as described in ref *14*. Fluorescent labeling of BSA with Cy5 or FITC was performed as described in ref *15*. Conjugation of labeled BSA to peptides derived from the NLS of SV40 T-antigen (CGGGPKKKRKVED) or from human PLSCR1 (CGGGKISKHWTGI) or its scrambled version (CGGKGSTHIGWIK) was performed as described in ref *15*. Unlabeled MBP−PLSCR1 and various deletion mutants of PLSCR1 fused to MBP were detected with monoclonal antibody (mAb) 4D2 raised against PLSCR1, which reacts with an epitope near the N-terminus of PLSCR1 (*5*) or with a rabbit polyclonal antibody (pAb) raised against MBP (New England Biolabs). Localization of Lamin A was detected by a rabbit pAb raised against human Lamin A (*16*). Import reaction mixtures contained either 2.5 mg/mL HeLa cytosol or recombinant factors at the following concentrations: 100 nM His-importin α, 62.5 nM importin *β*, 450 nM Ran, and 500 nM NTF2. The import reaction mixture was supplemented with an energy-regenerating system and 1 mM GTP (see ref *15* for details).

Cell Culture and Transient Transfection. Mouse fibroblast cell line SVT2 was cultured in DMEM (Mediatech) containing 10% fetal bovine serum (Sigma). Transient transfection was performed in SVT2 cells using Lipofectamine Plus transfection reagent (Gibco Invitrogen Corp.), according to the manufacturer's instructions.

Microtiter Plate Binding Assay. Solid phase binding assays were carried out on microtiter plates (Maxisorp, Nunc) coated with 25 ng of MBP−PLSCR1, MBP−PLSCR1(1−98), or His-importin α. Assays were conducted as previously described (*17*). Binding of His-importin α to MBP, MBP−PLSCR1, or MBP−PLSCR1(1−98) immobilized on microtiter plates was detected by an affinity-purified rabbit pAb raised against human His-importin α. Binding of MBP−PLSCR1 or MBP−PLSCR1(1−98) to His-importin α immobilized on microtiter plates was detected by anti-PLSCR1

mAb 4D2. Horseradish peroxidase-conjugated secondary antibodies were used for colorimetric detection (Pierce Chemical Co.) using 3,3′,5,5′-tetramethylbenzidine as the substrate (Calbiochem).

PLSCR1−Genomic DNA Binding. Four milligrams of the bovine thymus genomic DNA−cellulose conjugate (Sigma) or cellulose-type 50 (Sigma) was incubated with 2 μg of purified recombinant MBP−PLSCR1 or MBP at 4 °C for 1 h in 20 mM Tris (pH 7.5), 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mg/mL BSA, 0.2% Triton X-100, and protease inhibitor cocktail. The beads were thoroughly washed with 20 mM Tris (pH 7.5), 110 mM potassium acetate, 10% glycerol, 1 mM EDTA, 1 mg/mL BSA, and 0.2% Triton X-100. Subsequently, the beads were equilibrated in 20 mM Tris (pH 7.5), and elution was done sequentially in phosphate-buffered saline, with increasing concentrations of NaCl (from 150 to 300 mM). The eluate was centrifuged at 960*g* for 1 min and the supernatant used for analysis. For Western blot analysis, proteins were resolved on a 10% Tris-glycine gel and transferred to a nitrocellulose membrane. MBP−PLSCR1 and MBP were detected with rabbit anti-MBP pAb (New England Biolabs).

RESULTS

Nuclear Import of PLSCR1 Is Mediated by the Importin α/β Nuclear Import Pathway and Is Ran-Dependent. To study the mechanism by which PLSCR1 enters the nucleus, an MBP−PLSCR1 construct was generated. This was done to increase the molecular mass of PLSCR1 to 77 kDa, well above the apparent diffusion limit of the NPC (less than ~40 kDa). A digitonin-permeabilized cell assay was carried out to test for requirements of temperature, energy, and cytosolic factors for nuclear import of PLSCR1. Substantial nuclear accumulation of PLSCR1 was detected when permeabilized cells were supplemented with cytosol and an energy-regenerating system at 30 °C (Figure 1A−C). Nuclear import of PLSCR1 was inhibited when the same reaction was carried out at 4 °C (Figure 1D−F) or when it was energy-depleted by treatment with hexokinase and glucose (Figure 1J−L). WGA, a lectin that binds to the NPC and that inhibits receptor-mediated transport, also inhibited the nuclear import of PLSCR1 (Figure 1G−I). For discussion of panels M−O, see below. Collectively, these results suggest that nuclear accumulation of PLSCR1 is mediated by an active nuclear transport mechanism.

Most signal-dependent nuclear transport is mediated by nucleocytoplasmic shuttling receptors of the importin β/karyopherin β family (for reviews, see refs *18* and *19*). NLSs or nuclear export signals (NESs) in proteins are recognized directly by transport receptors or indirectly via adaptor proteins that bind to the receptors. After a receptor−cargo complex is formed, it is translocated through the NPC until it reaches the nucleus where the cargo is released from the receptor and the latter is recycled. The requirement for importin α and importin β for nuclear import of PLSCR1 was studied in digitonin-permeabilized cells (Figure 2). Nuclear import of PLSCR1 was observed when the permeabilized cells were supplemented with exogenous recombinant importin α/β (Figure 2A), and the level was considerably diminished when either importin α or importin β was omitted (panel B or C of Figure 2, respectively). Inhibition

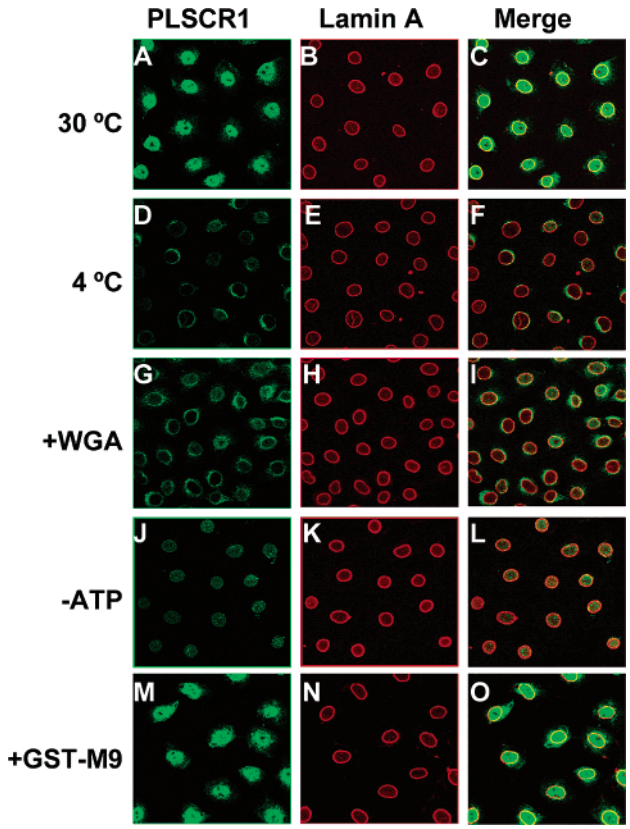

FIGURE 1: Dependence of the nuclear distribution of PLSCR1 on cytosol, temperature, and energy, and inhibition of nuclear uptake by WGA. Digitonin-permeabilized NRK cells were incubated with 8 pmol of MBP−PLSCR1 for 20 min at 30 °C (A) or 4 °C (D) in the presence of cytosol and an energy-regenerating system. For panel G, 8 μg of WGA was added. For panel J, the ATP-regenerating system was replaced with an ATP-depleting system (0.8885 unit of hexokinase and glucose). For panel M, a 10-fold molar excess of the transportin cargo protein GST-M9 was added. Panels B, E, H, K, and N are the same cells labeled with pAb against Lamin A. Panels C, F, I, L, and O are merged images of PLSCR1 and Lamin A. The cellular localization of PLSCR1 was determined by immunofluorescence with mAb 4D2 and a FITC-labeled secondary antibody and that of Lamin A with an anti-Lamin A and a Cy5-labeled secondary antibody. Samples were visualized by confocal microscopy.

of nuclear import of PLSCR1 was also observed in the presence of WGA (data not shown) or in the presence of known competitive inhibitors of this transport pathway, including a 10-fold molar excess of another importin α cargo protein [BSA coupled with the NLS peptide derived from the SV40 T-antigen (Figure 2E)]. By contrast, nuclear import of PLSCR1 was not inhibited by a 10-fold molar excess of a cargo (GST-M9) of another major nuclear import receptor, transportin (Figure 1M−O).

The small GTPase Ran, which interacts with importin β-type receptors and thus regulates their association with cargo, plays a key role in driving both nuclear import and export (*19*). Import receptors bind their cargo in the cytosol. After the complex enters the nucleus, import cargo is released through receptor binding to RanGTP. Conversely, export receptors form complexes with cargo and RanGTP in the nucleus. After translocation through the NPC, RanGAP-mediated hydrolysis occurs, resulting in dissociation of export complexes. Whereas nuclear import of MBP−PLSCR1 was observed in the fully reconstituted transport system containing Ran (Figure 2A), import of MBP−PLSCR1 was inhibited

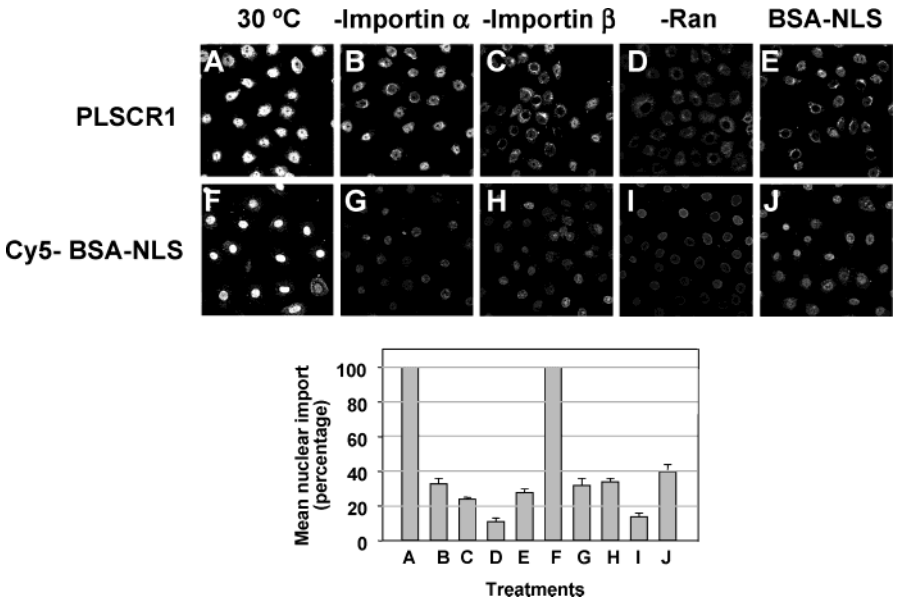

FIGURE 2: Role of the importin α/*β* nuclear import pathway in the nuclear distribution of PLSCR1. Digitonin-permeabilized NRK cells were incubated with 8 pmol of MBP−PLSCR1 (A−E) or 8 pmol of Cy5−BSA−NLS (containing the peptide sequence derived from the NLS of SV40 T-antigen) (F−J), and nuclear import was conducted with recombinant factors as described in Experimental Procedures. For panels A and F, nuclear import of MBP−PLSCR1 and Cy5−BSA−NLS was performed at 30 °C. For panels B and G, importin α was omitted. For panels C and H, importin *β* was omitted. For panels D and I, Ran was omitted. For panels E and J, MBP−PLSCR1 and Cy5−BSA−NLS competed with a 10-fold molar excess of unlabeled BSA−NLS. MBP−PLSCR1 was detected as described in the legend of Figure 1. Samples were visualized by confocal fluorescence microscopy. The bar chart is a digital quantitation of the images by NIH Image 1.62.

in the absence of Ran (Figure 2D) or in the presence of a mutant form of Ran (RanQ69L, data not shown) that is insensitive to RanGAP and therefore is predominantly in the GTP-bound form (*20*). Nuclear import of MBP−PLSCR1 was also inhibited in the presence of an importin *β* mutant that lacks its Ran binding site (deletion of residues 1−44, data not shown). These results suggest that just as in the conventional nuclear import pathway, Ran is specifically required for the nuclear import of PLSCR1.

Identity of the Segment of PLSCR1 Required for Its Nuclear Import. Since the sequence of PLSCR1 does not contain a sequence homologous to the classical NLS of SV40 T-antigen, we analyzed various deletion mutants of this protein fused to MBP to exceed the diffusion limit of the NPC, to identify the segment of PLSCR1 that is required for its nuclear import (Figure 3A). Deletion of the 211 N-terminal residues of PLSCR1 (construct 212−318 in Figure 3A,B) did not compromise its ability to be imported into the nucleus. By contrast, truncation of the C-terminus of PLSCR1 (deletion of residues 242−318; see construct 1−241 in Figure 3A,B) rendered the protein inactive for nuclear import. Because both PLSCR1(1−290) and PLSCR1-(212−290) were actively imported into the nucleus, and PLSCR1(1−241) was not, these results implied that the functional NLS of the protein was contained between residues 242 and 290. The region within the sequence of residues 242−290 revealing the largest cluster of positively charged amino acids was ^{257}GKISKHWTGI^{266}. To test whether this region has NLS activity, a peptide corresponding to these amino acids was synthesized and conjugated to BSA for testing in a nuclear import assay. Figure 4B shows that FITC−BSA−PLSCR1(257−266) was concentrated in the nucleus in an energy-dependent and WGA-sensitive manner, whereas a scrambled version of this peptide was not. Moreover, nuclear import of FITC−BSA−PLSCR1(257−266) competed with a 10-fold molar excess of MBP−PLSCR1, and nuclear import of MBP−PLSCR1 competed with a 10-fold molar excess of FITC−BSA−PLSCR1(257−266). To test whether this region contains the NLS in the context of full-length PLSCR1, positively charged amino acids within this segment (^{257}GKISKHWTGI^{266}) were mutated in addition to the mutation of the palmitoylation site to create the PLSCR1(^{184}AAAPAA^{189},^{257}GAISAAWTGI^{266}) mutant, and SVT2 cells were transiently transfected with this mutant. Figure 5 shows that mutations in the NLS region blocked the ability of PLSCR1 to localize in the nucleus. Nuclear localization of the PLSCR1 construct with mutation in the Cys string palmitoylation site, and the absence of nuclear localization of the PLSCR1 construct with mutation in the NLS region, were observed for all cells that were examined regardless of the apparent level of protein expression, suggesting that failure of the NLS region mutant to undergo nuclear uptake was unrelated to any potential difference in protein expression or stability. As was noted, the PLSCR1(257−266) peptide supported nuclear import and competed with import of full-length PLSCR1 (Figure 4), whereas mutation within this same segment in full-length PLSCR1 eliminated nuclear import of this protein (Figure 5). We therefore propose that this segment comprises its NLS.

PLSCR1 Interacts Directly with Importin α. To confirm that the observed nuclear import of PLSCR1 by the importin α/*β* transport system entails a direct interaction of PLSCR1 with importin α, we next evaluated potential binding interactions between these two recombinant proteins by solid phase binding assays. As shown in Figure 6, His-importin α showed saturable binding to immobilized MBP−PLSCR1 (panel A) and MBP−PLSCR1 showed saturable binding to immobilized His-importin α (panel B), with apparent affinities (mean ± the standard deviation) of 40 ± 4.5 and 27.8

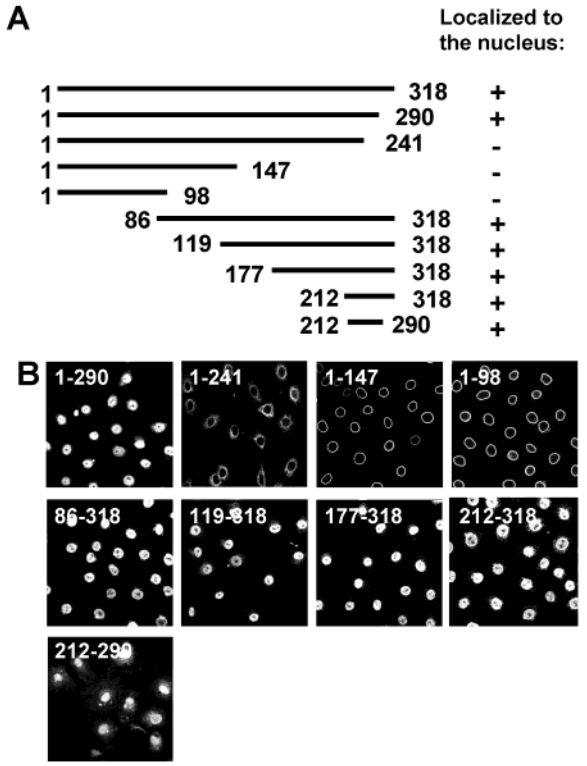

FIGURE 3: Mapping of a region in PLSCR1 required for nuclear import. Digitonin-permeabilized NRK cells were incubated with 7.3 pmol of each of the MBP−PLSCR1 mutants, cytosol, and an energy-regenerating system (see Experimental Procedures). (A) Schematic representation of the deletion mutants compared to full-length PLSCR1 with a summary of whether nuclear import was observed (+) or not observed (−). (B) Confocal fluorescence images of the various MBP−PLSCR1 constructs depicted in panel A. The cellular localization of MBP−PLSCR1 and MBP−PLSCR1 deletion mutants PLSCR1(86−318), PLSCR1(119−318), PLSCR1-(177−318), PLSCR1(212−318), and PLSCR1(212−290) were determined by immunofluorescence with pAb against MBP and a FITC-labeled secondary antibody. MBP−PLSCR1 deletion mutants PLSCR1(1−290), PLSCR1(1−241), PLSCR1(1−147), and PLSCR1-(1−98) were visualized with mAb 4D2 and a FITC-labeled secondary antibody as described in the legend of Figure 1. All assays were performed at 30 °C. Data are from a single experiment that is representative of at least five identical experiments.

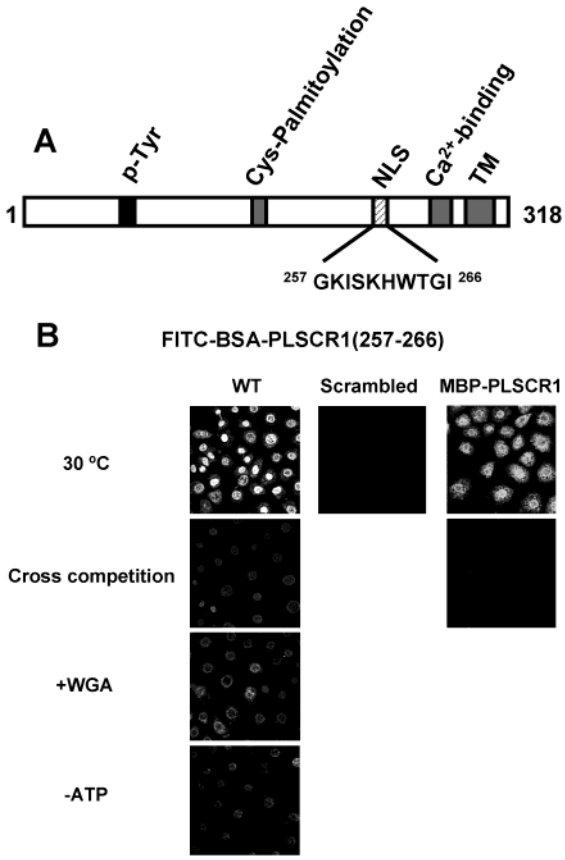

FIGURE 4: Mapping of the region in PLSCR1 containing the putative NLS. (A) Schematic representation of the putative NLS region in PLSCR1 and the sequence deduced from the data from Figure 3. Other domains described in the figure are as follows: p-Tyr, sites of phosphorylation by c-Abl and c-Src kinases at Y^{69} or Y^{74}, sites of Cys palmitoylation (residues ^{184}CCCPCC^{189}), Ca^{2+}-binding sites (residues D273−D284), and TM putative transmembrane domain (residues K288−E306). (B) From top to bottom, confocal fluorescence images of either FITC−BSA−PLSCR1(257−266) or FITC−BSA−PLSCR1 scrambled peptide or MBP−PLSCR1 when nuclear import was performed with the fully reconstituted import system, in the presence of a 10-fold molar excess of MBP−PLSCR1 [added to compete with the import of FITC−BSA−PLSCR1(257−266)] or a 10-fold molar excess of FITC−BSA−PLSCR1(257−266) (added to compete with the import of MBP−PLSCR1), in the presence of 8 μg of WGA, or in the presence of an ATP-depleting system (see Experimental Procedures). Data are from a single experiment that is representative of three experiments. MBP−PLSCR1 was visualized by confocal fluorescence microscopy using pAb against MBP detected with a Cy5-labeled secondary antibody.

± 2.6 nM, respectively. By contrast, MBP−PLSCR1(1−98), a deletion mutant that did not exhibit nuclear import (Figure 3), did not bind to His-importin α (Figure 6).

PLSCR1 Is Retained in the Nucleus Even after Disruption of the Nuclear Membrane but Is Released after Digestion of Nuclear DNA. As shown in Figure 7, once PLSCR1 is imported into the nucleus, we did not observe subsequent release of it from the nucleus (panels A and F). Furthermore, PLSCR1 was retained in the nucleus after disruption of the nuclear envelope with Triton X-100 in physiological buffer, a condition that liberates another nuclear localized cargo (the NLS of SV40 T-antigen; panels B and G, respectively). Elution of PLSCR1 was achieved only by subsequently increasing NaCl concentrations (in phosphate buffer saline) to >150 mM (panels C−E), implying its potential retention in the nucleus through electrostatic interactions, possibly with nuclear DNA. This is further supported as shown in Figure 8 (panels G−I). As the data indicate, digestion of nuclear DNA with DNase resulted in complete release of PLSCR1 from the nucleus. This suggests that nuclear retention of PLSCR1 reflects interactions with genomic DNA, either directly or through another DNA-bound constituent.

PLSCR1 Interacts with Genomic DNA. Since the retention of PLSCR1 in the nucleus appeared to be able to be attributed to electrostatic interactions, and its retention was also alleviated by DNase treatment, we next considered whether this protein might directly bind to DNA. As shown in Figure 9, PLSCR1 was confirmed to bind directly to purified human genomic DNA and, once bound, required 250 mM NaCl to be eluted, which is similar to what was observed for its extraction from the nucleus when imported *in vitro*.

DISCUSSION

PLSCR1 is a multiply palmitoylated, lipid raft-associated protein that is normally localized to the plasma membrane,

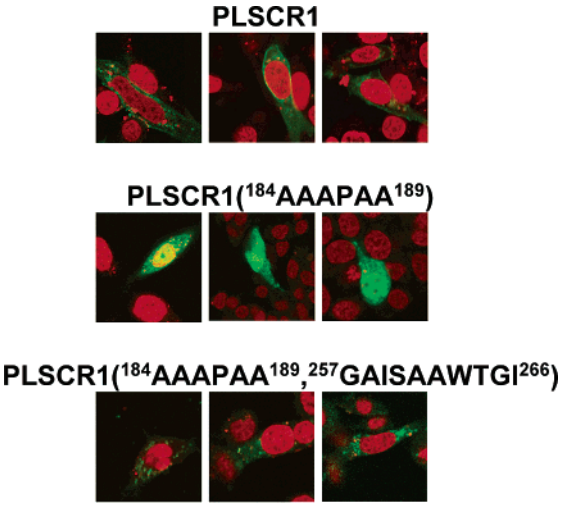

FIGURE 5: Mutation of putative NLS blocks nuclear import of PLSCR1. Confocal fluorescence images of murine SVT2 fibroblasts transiently transfected with (from top to bottom) pcDNA3 containing cDNA for wild-type human PLSCR1 or the PLSCR1-(^{184}AAAPAA^{189}) and PLSCR1(^{184}AAAPAA^{189},^{257}GAISAAWTGI^{266}) mutants. Expressed human PLSCR1 antigen was detected with mab 4D2 and FITC-conjugated goat anti-mouse IgG. Following immunostaining and DNA labeling with propidium iodide, cells were visualized by confocal fluorescence microscopy. Data are from a single experiment that is representative of two.

where it has been implicated to participate in the reorganization of the plasma membrane PLs that accompanies cell activation, injury, or apoptosis. The recent finding of its localization to the nucleus in the absence of palmitoylation, or following induction of new PLSCR1 synthesis in response to cytokine treatment (*7*), raises the following questions: (1) which mechanism(s) does PLSCR1 employ to enter the nucleus, and (2) what biologic function does this nucleus-distributed PLSCR1 play?

Receptor-mediated nuclear transport plays a central role in the proper regulation and trafficking of nuclear proteins involved in transcription, DNA replication, and chromatin remodeling. The observation of nuclear import of PLSCR1 is therefore of particular interest in light of recent evidence of a distinct role for PLSCR1 in the overall cellular responses to a variety of cytokines and growth factors, including, specifically, in the regulation of cell proliferation and cell maturation (*8*, *21*, *22*).

As a first step toward identifying the potential biologic significance of nucleus-distributed PLSCR1, we undertook to determine the molecular mechanism by which this protein enters the nucleus and to identify the component(s) of the nucleus with which PLSCR1 might interact. The mechanism of nuclear entry of PLSCR1 was of particular concern as (1) its molecular mass of ~37 kDa is close to the apparent limit of ~40 kDa for diffusion through the NPC (*11*, *19*), (2) the polypeptide does not contain a predicted conventional NLS, and (3) PLSCR1 might itself perturb the integrity of the nuclear envelope, through its interactions with membrane PL.

In the study presented here, we have demonstrated that the nuclear import of PLSCR1 is dependent on energy, temperature, and a specific segment of the polypeptide (residues 242−290) located near the carboxy terminus (Figure 4). Further, we show that PLSCR1 is imported into the nucleus in an importin α/β-, GTP-, and Ran-dependent

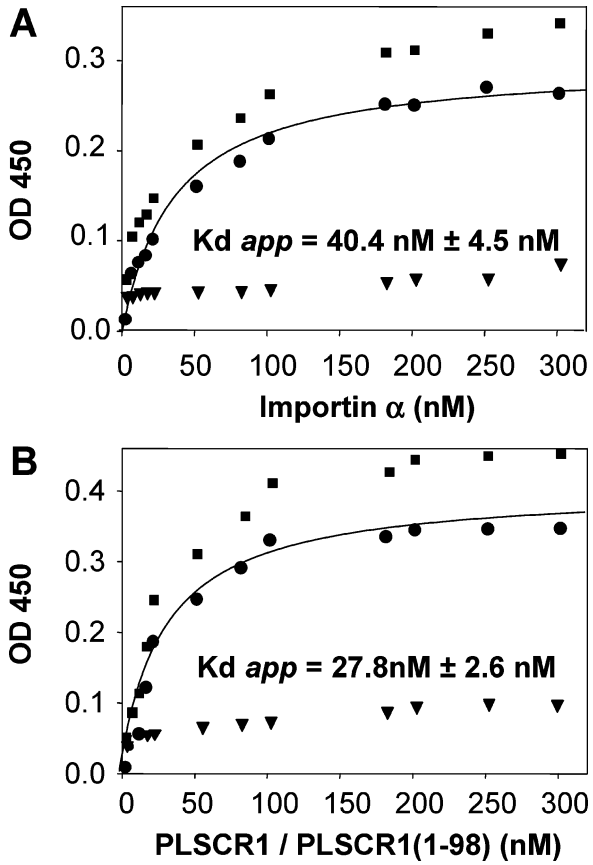

FIGURE 6: Direct interaction between PLSCR1 and importin α. Characterization of the binding of MBP−PLSCR1 to His-importin α. (A) Increasing concentrations of His-importin α were incubated with immobilized MBP−PLSCR1 or MBP−PLSCR1(1−98), and the amount of bound His-importin α was measured (see Experimental Procedures). (B) Increasing concentrations of MBP−PLSCR1 or MBP−PLSCR1(1−98) were incubated with immobilized His-importin α, and the amount of bound MBP−PLSCR1 was measured with mAb 4D2 (see Experimental Procedures): MBP−PLSCR1 (■) and MBP−PLSCR1(1−98) (▼). In both panels, circles represent the correction for nonspecific binding obtained by subtracting data for MBP−PLSCR1(1−98) from data for MBP−PLSCR1. Results are from duplicates of a single experiment. The corrected data (●) were fitted to the equation $B(Y) = (B_{max}Y)/(K_d + Y)$, where Y is the concentration of His-importin α (A) or MBP−PLSCR1 (B) and B is the amount of His-importin α (A) or MBP−PLSCR1 (B) specifically bound. The correlation coefficients for fitting the data to the curves were >0.99. The derived apparent K_d's are expressed as the mean ± the standard deviation.

manner (Figure 2). Our data also indicate that PLSCR1 can itself directly bind to importin α, and that this interaction occurs with an apparent affinity that is consistent with that observed for other known importin α cargo proteins (Figure 6 and ref *23*). Examination of a synthetic peptide encompassing residues 257−266 by nuclear import and competition studies suggested that this segment contains the PLSCR1 NLS. This was further supported by the finding that point mutations in the NLS region rendered full-length PLSCR1 inactive in nuclear import in intact SVT2 cells. This NLS is distinct from the prototypical monopartite NLS defined by a cluster of five basic residues (Table 1). We found that after nuclear import, the removal of PLSCR1 from the nucleus required sequential treatment with Triton X-100 and elevated salt concentrations, or DNase treatment, suggesting some electrostatic interaction with a chromosomal (genomic DNA or another protein bound to genomic DNA) component(s).

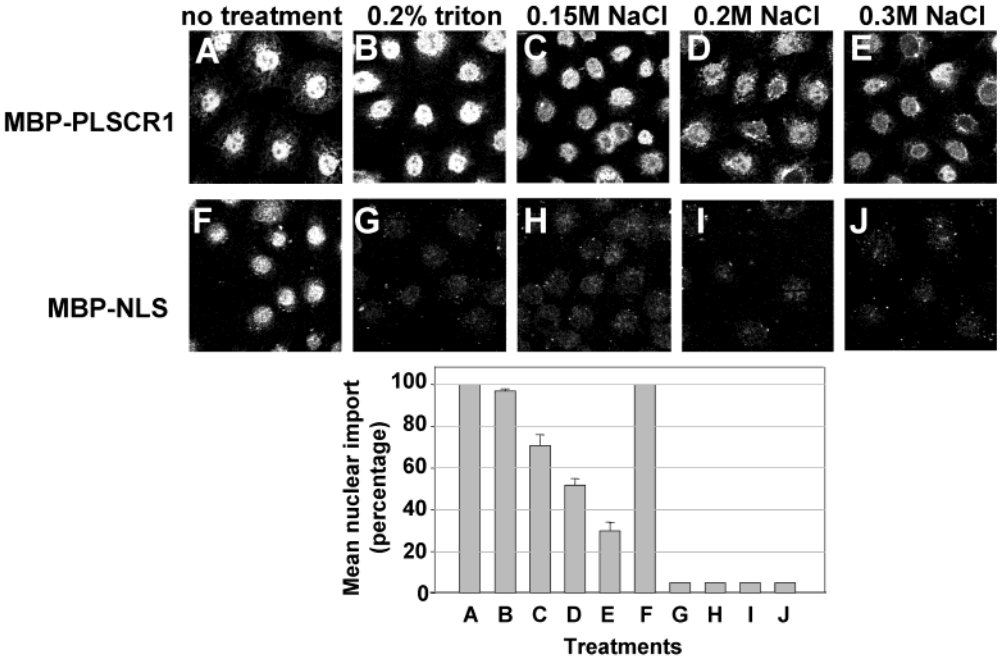

FIGURE 7: Effect of Triton X-100 and NaCl on nuclear retention of PLSCR1. Digitonin-permeabilized NRK cells were incubated with 8 pmol of MBP−PLSCR1 or MBP−NLS (derived from SV40 T-antigen) in the presence of cytosol and an energy-regenerating system to promote nuclear import. For panels A and F, after the nuclear import assay, cells were washed thoroughly, incubated for 5 min in transport buffer, and subsequently fixed and immunostained using pAb against MBP (see Figure 3). For panels B and G, before fixation and immunostaining, the cells were incubated (10 min at room temperature) with 0.2% Triton X-100 in transport buffer and washed thoroughly with transport buffer. For panels C and H, following treatment with Triton X-100 and washing with transport buffer, cells were washed and incubated (10 min at room temperature) with 150 mM NaCl and 10 mM sodium phosphate (pH 7.5) followed by fixation and immunostaining. Panels D and I are as described above, substituting 0.2 M NaCl. Panels E and J are as described above, substituting 0.3 M NaCl. Following immunostaining, cells were visualized by confocal fluorescence microscopy. Data are from a single experiment that is representative of five experiments. The bar chart is a digital quantitation of the images by NIH Image 1.62.

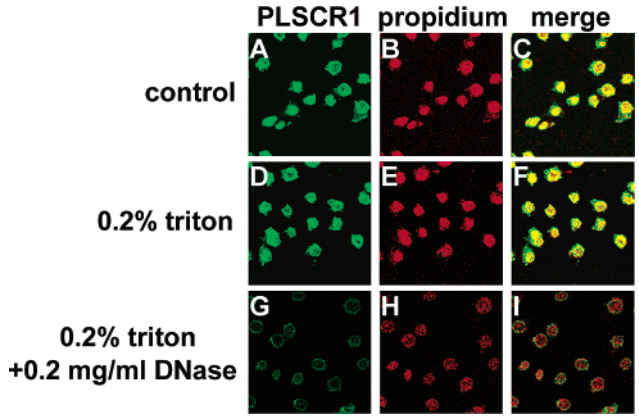

FIGURE 8: Effect of DNase treatment on nuclear retention of PLSCR1. Digitonin-permeabilized NRK cells were incubated with 8 pmol of MBP−PLSCR1 in the presence of cytosol and an energy-regenerating system to promote nuclear import. (A) After the nuclear import assay, cells were washed thoroughly, incubated for 5 min in transport buffer, and subsequently fixed and immunostained using pAb against PLSCR1 (see Figure 1). (D) Before fixation and immunostaining, the cells were incubated (20 min at 30 °C) with 0.2% Triton X-100 in DNase buffer and washed thoroughly with transport buffer. (G) Before fixation and immunostaining, the cells were incubated (20 min at 30 °C) with 0.2% Triton X-100 and 0.2 mg/mL DNase in DNase buffer and washed thoroughly with transport buffer. Panels B, E, and H show the same cells, nuclear DNA stained with propidium iodide. Panels C, F, and I are merged images of PLSCR1 and nuclear DNA. Following immunostaining and DNA labeling with propidium iodide, cells were visualized by confocal fluorescence microscopy. Data are from a single experiment that is representative of two.

This led us to study its interaction with genomic DNA, to which PLSCR1, an acidic protein (calculated pK_a of 4.85), was found to bind, and which required an elevated salt concentrations to elute.

Whereas import of a protein into the nucleus is generally considered to involve specific nuclear localization signals, it is becoming increasingly clear that efficient and well-controlled import of proteins that lack a canonical NLS also occurs. Interestingly, despite the observation that PLSCR1 interacts directly with importin α with a high apparent affinity [similar to that reported for the SV40 T-antigen NLS (*24*)], requires importin *β* and Ran for nuclear import, and thus shows functional similarity to the conventional NLS, there are some fundamental differences in terms of amino acid composition and sequence. The classical SV40 T-antigen NLS is lysine-rich, whereas the putative PLSCR1 NLS we identify has two lysines separated by two neutral amino acids and one histidine (Figure 4 and Table 1). This sequence in PLSCR1 resembles the c-*myc* NLS (*25*), in which only three of nine amino acids are basic, yet all regions of the signal are either essential or important for nuclear entry. One can speculate that since the NLS derived from SV40 T-antigen was found to compete with PLSCR1 for nuclear import (see Figure 2), they must bind to similar or overlapping regions in importin α.

Shuttling proteins continuously move between the cytoplasm and nucleus. The steady-state localization of these proteins reflects a dynamic process of nuclear entry and exit. Therefore, localization of a protein would be dependent on the strength of its NLS versus its NES. In the case of PLSCR1, our data suggest that PLSCR1 is imported into the nucleus, and then retained through electrostatic interactions with an immobile nuclear component even after

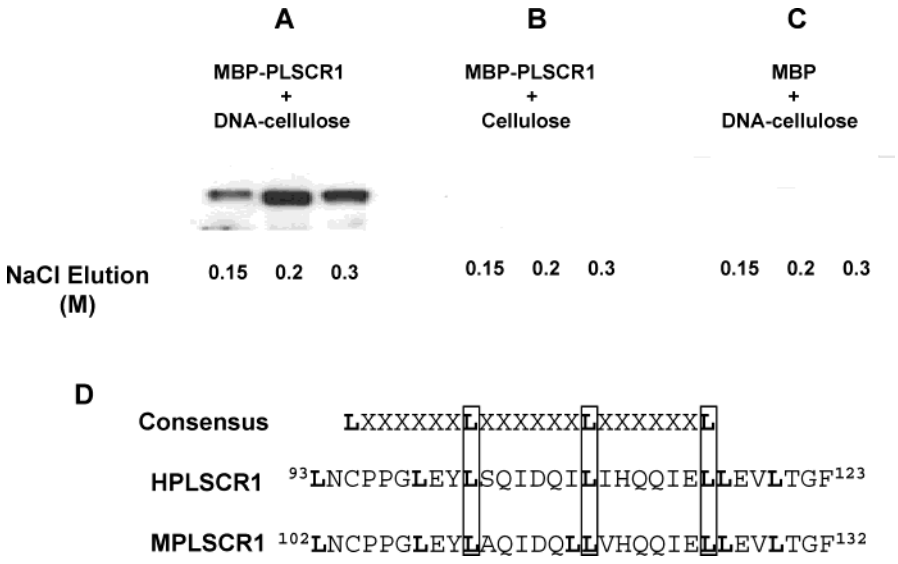

FIGURE 9: Binding of PLSCR1 to genomic DNA. The genomic DNA−cellulose conjugate or unconjugated cellulose was incubated with MBP−PLSCR1 or MBP as described in Experimental Procedures. The cellulose was then extensively washed and bound protein eluted at the indicated salt concentrations. The eluted proteins were resolved on 10% Tris-glycine gel, and Western blotting was performed using pAb anti-MBP (see Experimental Procedures): (A) elution of MBP−PLSCR1 from the DNA−cellulose conjugate, (B) elution of MBP−PLSCR1 from cellulose control, (C) elution of MBP from DNA cellulose conjugate, and (D) sequence from human and mouse PLSCR1 that is 75% similar to a consensus sequence of a leucine zipper according to the PROSITE database (see the Discussion). Elution was carried out at pH 7.5 in 10 mM sodium phosphate at the indicated NaCl concentrations. Data are from a single experiment that is representative of two.

Table 1: NLS Sequences of PLSCR1, SV40 T-Antigen, and c-*myc*

Signal Source	Signal Sequence
SV40 T-antigen	**PKKKRKV**
c-*myc*	**PAAKRVKLD**
PLSCR1	**GKISKHWTGI**

detergent lysis of the nuclear envelope (Figure 7). Whereas our data provide no evidence for a directed nuclear export of PLSCR1 through the NPC, the possibility that this might occur in the intact cell cannot now be excluded, particularly in light of the phosphorylations, thiol esterification, and other post-translational modifications to PLSCR1 that are known to occur *in situ* (*1*).

As noted above, PLSCR1 is known to itself be transcriptionally induced by the interferons and by a variety of growth factors, and it is under such conditions of cytokine-induced synthesis that a marked increase in nuclear trafficking of this protein has been observed (*10*). Furthermore, there is now substantial evidence which suggests that this protein plays some role in the overall cellular response to these same cytokines and growth factors, including direct effects on both growth factor and cytokine-regulated cell proliferation and maturation (*8*, *21*, *22*). As noted, we observed that PLSCR1 is specifically and actively imported into the nucleus, where it may be retained by binding to DNA (Figures 7 and 9) or released by DNase treatment (Figure 8). It is therefore of interest to note that computer analysis of potential motifs in the PLSCR1 polypeptide predicts a partial leucine zipper (between PLSCR1 residues 93 and 123; see Figure 9D), a common protein dimerization motif that is found in many transcription factors. This raises the intriguing possibility that in addition to its other known or suspected activities at the plasma membrane, this protein might itself either function as a transcription factor within the nucleus or otherwise serve to modify gene transcription, for example, by competing with one or more nuclear transcription factors from their DNA binding sites.

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