Members of the phospholipid scramblase (PLSCR) family play active roles in altering lipid asymmetry at the plasma membrane including phosphatidylserine (PtdSer) exposure on the cell surface. To determine whether PtdSer biosynthesis and externalization are altered by PLSCR activities during apoptosis, Chinese hamster ovary K1 lines stably overexpressing PLSCR1 and PLSCR2 were established. PLSCR1 was localized on the plasma membrane, whereas PLSCR2 was predominantly in the nucleus. Cells overexpressing PLSCR1 showed suppressed growth, altered cell morphology, and higher basal levels of cell death. Following UV irradiation, these cells showed earlier and enhanced PtdSer exposure, increased caspase-3 activation, apoptotic nuclear changes, and PARP cleavage indicative of apoptosis. UV irradiation in cells overexpressing PLSCR1 led to a 4-fold stimulation of PtdSer synthesis (accompanied by increased movement of newly made PtdSer into microvesicles) relative to untreated PLSCR1 cells, whereas PtdSer formation in UV-irradiated vector control cells increased only by 2-fold. No differences in these responses were observed between PLSCR2-expressing cells and vector controls. PtdSer synthesis and its transbilayer movement stimulated by PLSCR1 overexpression were blocked by a caspase inhibitor along with progression of apoptosis. Thus, our studies showed that overexpression of PLSCR1 in Chinese hamster ovary K1 cells stimulated caspase-dependent PtdSer externalization and synthesis, implying an up-regulation of PtdSer formation in response to enhanced outward movement of this phospholipid to the cell surface during apoptosis. PLSCR1 also appears to influence progression of UV-induced apoptosis and could be a point of regulation or intervention during programmed cell death.

Phosphatidylserine (PtdSer)\(^1\) externalization is observed during cell activation, aging, and apoptosis (1–3). This membrane lipid rearrangement may have various roles depending on the cell type and conditions for mobilization of PtdSer (4). When exposed on the cell surface during programmed cell death, PtdSer signals the removal of apoptotic cells to avoid inflammatory reactions (5, 6). Phospholipid scrambling also leads to inward movement of sphingomyelin (SM), where it may be hydrolyzed by neutral sphingomyelinase; subsequent production of ceramide may play an important role in membrane blebbing during apoptosis (7). Mechanisms underlying PtdSer externalization are unclear, but concomitant inactivation of aminophospholipid translocase activity and activation of phospholipid scramblase (PLSCR) as a result of rising concentrations of cytoplasmic calcium are considered to play a major role (8–10). Aminophospholipid scramblase helps to maintain membrane asymmetry by moving PtdSer and PtdEtn on the outer leaflet back to the inner membrane bilayer (11–13). Phospholipid scramblase, when activated, catalyzes bidirectional movement of all of the membrane phospholipids (14–16).

A family of phospholipid scramblases, including HuPLSCR 1–4 and their murine orthologs, MuPLSCR 1–4, as well as a rat PLSCR homolog to HuPLSCR1, has been identified (17, 18). PLSCR proteins have a short extracellular domain or no extracellular domain, whereas their intracellular domains are highly variable in length and composition (18). Expression of PLSCR1 is induced at the transcriptional level by interferon, indicating its potential involvement in interferon-mediated activities (19, 20). The PLSCRs are type II transmembrane proteins that are highly conserved in the calcium-binding C-terminal domain (21). Calcium binding to the proteins is presumed to induce a major conformational change and activate the lipid scrambling activity (22). Some members contain PXXP and PPXY motifs, indicating potential interaction with signaling molecules that contain SH3 or WW domains (17, 23). PLSCR1 binds to the SH3 domain of c-Abl tyrosine kinase and is constitutively tyrosine phosphorylated by this enzyme (24). PLSCR1 is also found to be enriched in lipid rafts and phosphorylated upon its interaction with epidermal growth factor receptor (25). Other post-translational modifications of PLSCR implicated in regulating its normal functions include palmitoylation at multiple cysteine residues (26) and phosphorylation at one threonine site through protein kinase C\(\beta\) (27). PLSCR proteins not only serve to activate membrane lipid scrambling but also play versatile roles in signaling pathways that regulate cellular events such as proliferation and oncogenic transformation (20, 24).

Our previous study using U937 cells showed that PtdSer biosynthesis increased markedly along with the exposure of PtdSer on the cell surface during apoptosis induced by various stimuli. Compared with other newly synthesized phospholipids, PtdSer moved preferentially into microvesicles budding from apoptotic cells. This stimulation of synthesis is regulated...
in a caspase-dependent manner (28). Normally, PtdSer synthesis occurs at the endoplasmic reticulum and mitochondria-associated membranes through base exchange of t-serine with the head group of other existing phospholipids (29), catalyzed by two isoforms of PtdSer synthase (PSS) with different substrate specificities. PSS I utilizes phosphatidylcholine, whereas PSS II converts PtdEtn to PtdSer (30). Newly synthesized PtdSer is then rapidly transported to other membranes including the plasma membrane. PtdSer also is delivered to mitochondria where it can be decarboxylated to form PtdEtn. Regulation of PtdSer biosynthesis and its movement in mammalian cells is not well understood. In CHO-K1 cells, feedback appears to regulate serine base exchange reactions to maintain constant levels of PtdSer. The cells incorporate exogenous PtdSer rapidly, blocking de novo synthesis of PtdSer by a competitive feedback mechanism (31–33).

We hypothesized that PtdSer exposure on the cell surface and subsequent movement into microvesicles may increase the need for more PtdSer as low intracellular PtdSer levels release feedback inhibition of PtdSer biosynthesis in response to the PtdSer scrambling activity. To examine a possible regulatory role of PLSCR, we established CHO cell lines overexpressing PLSCR1 and PLSCR2 isoforms and monitored biosynthesis of serine-derived phospholipids and their movement into the vesicles during UV-induced apoptosis. Our studies suggest that PLSCR activity influences both synthesis and reorientation of PtdSer in an isoform-specific and caspase-dependent manner during apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-c-Myc mAb was purchased from Clontech. Anti-human poly(ADP-ribose) polymerase (PARP) pAb was from Santa Cruz Biotechnology. Anti-ACTIVATED-caspa-se-3 pAb was from Promega. LipofectAMINE 2000 was obtained from Invitrogen. Annexin-V-FLUOS staining kit was from Roche Molecular Biochemicals. Trypan blue solution and propidium iodide (PI) were obtained from Sigma. z-VAD-fmk was purchased from Calbiochem. l-(1H)G/Serine, [methyl-3H]choline chloride, and [1,2-3H]choleolamine hydrochloride were obtained from Mäandel Scientific.

**Cell Culture**—Strain CHO-K1 was obtained from the American Type Culture Collection. The cells were maintained in a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum (CANSERA) and 300 μg/ml of hygromycin/ml and 200 μg/ml of G418/ml. Cell cultures were incubated at 37° C.

**Cloning of Phospholipid Scramblases into pcDNA3.1/Myc-His** 
A Expression Vector—Murine PLSCR1 cDNA (GenBank™ accession number AF159593) was kindly provided by Dr. Peter Sims (17, 34). Murine PLSCR2 homolog cDNA was obtained from ATCC (expression number AF015790) (21). PCR-amplified DNA fragments were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen) and checked by DNA sequencing. Full coding sequences were subcloned into the pcDNA3.1/Myc-His (+) A expression vector (Invitrogen) to generate the expression plasmids pcDNA-PLSCR1 and pcDNA-PLSCR2.

**Transient Transfection of CHO-K1 Cells**—CHO-K1 cells grown to 80–90% confluence were transfected with pcDNA-PLSCR1 or pcDNA-PLSCR2 constructs (1 μg of plasmid DNA) using LipofectAMINE 2000 reagent according to the manufacturer's instructions. The transfected cells were grown for 24 h before further analysis.

**Establishment of Stable Expression Clones**—To increase the effectiveness of selection, CHO-K1 cells were co-transfected with empty pTK-Hyg vector (Clontech) and pcDNA-PLSCR1 or pcDNA-PLSCR2 constructs according to the manufacturer's instructions. One day after LipofectAMINE 2000 transfection, selection medium containing 350 μg of G418/ml and 200 μg of hygromycin/ml was added to the cells, and the medium was refreshed every 48 h after the initial addition of antibiotics. Cell death was observed by 3 days after adding antibiotics. Stable clones were obtained by dilution subcloning and characterized by immunofluorescence and Western blotting. The cells overexpressing PLSCR1, PLSCR2, and vector control clone were maintained in regular growth medium containing 350 μg of G418/ml and 200 μg of hygromycin/ml.

**Western Blotting**—Cells were washed with cold Tris-buffered saline (TBS). For PARP preparations, the cell extracts were collected by lysing 1 × 10⁶ cells in 200 μl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 3.3% β-mercaptoethanol, 12.5% glycerol, 0.05% bromophenol blue). For scramble preparations, total protein extracts were prepared by lysing cells in 0.5 ml of lysis buffer (1% Triton X-100, 40 μl of protease inhibitor mixture (Roche Molecular Biochemicals) in TBS) followed by incubation on ice for 10 min. The cell lysate was centrifuged at 15,000 × g for 10 min at 4° C. Protein in a 200-μl aliquot was precipitated with 1 ml of cold acetone. The protein concentration of each sample was determined using a micro bca protein assay kit (Pierce). The samples were resolved by SDS-PAGE (8% for PARP and 10% for Myc tagged PLSCR1 and PLSCR2) and transferred to polyvinylidene difluoride membrane (Millipore) according to the manufacturer's instructions. For PARP antibody blotting, the membrane was incubated for 1 h with hybridization solution containing anti-human PARP (1: 4,000) in 5% skim milk with TTBS (0.04% Tween 20 in TBS). The blot was rinsed with TBS and incubated for 45 min with goat anti-rabbit horseradish peroxidase-coupled secondary antibody (1:10,000) in 5% skim milk with TTBS. For detection of Myc-tagged proteins, the membrane was incubated with hybridization solution containing anti-c-Myc mAb (1:2,000) according to the manufacturer's instructions. The blot was incubated with goat anti-mouse horseradish peroxidase-coupled secondary antibody (1:10,000). Enhanced chemiluminescence (Amersham Biosciences) was used to detect relevant proteins according to the manufacturer's instructions.

**Trypan Blue Exclusion Assay**—The cells were exposed to UV light for 10 min and cultured for 8 h. The attached cells were released using 0.25% trypsin and combined with the culture medium containing floating dead cells. The cells were pelleted by centrifugation at 1,000 × g for 10 min and were resuspended in PBS in the presence of an equal volume of trypan blue solution (0.4%, w/v); the percentage of blue cells/totals cells was counted using a hemocytometer.

**Immunofluorescence and Confocal Microscopy**—The cells were grown on coverslips. After induction, the cells were fixed with formaldehyde (3%, v/v) and permeabilized with 0.05% (w/v) Triton X-100 in PBS for 10 min at 20° C. The cells were incubated with anti-c-Myc mAb (1:500, v/v) in PBS containing 1% BSA (PBS-BSA) overnight at 4° C and rinsed with PBS-BSA. Alexa flour 488-conjugated goat anti mouse secondary antibody (Molecular Probes) was added (2 μg/ml) and incubated for 45 min at 22° C. The cells were rinsed twice with PBS-BSA. The coverslips were mounted in 2.5%, 1,4-diazabicyclo (2,2,2)octane and 90% glycerol in 50 mM Tris-HCl (pH 9.0) on glass slides. Detection of activation of caspase 3 was performed with overnight incubation with anti-ACTIVATED-caspa-se-3 pAb (1:500, v/v) at 4° C followed by staining with Alexa flour 488 goat anti-rabbit IgG at 22° C. The coverslips were rinsed with PBS and incubated with goat anti-rabbit IgG conjugated to Alexa flour 488 (1:500, v/v) for another 15 min. Alexa flour 488 staining was visualized with excitation at 488 nm, and PI staining was visualized with excitation at 543 nm using a Zeiss inverted laser-scanning confocal microscope, LSM-510. Superimposed images were obtained with LSM-510 Image software.

**Labeling Phospholipids with Radioactive Precursors, Lipid Extraction, and Phosphorous Assay**—After incubation of cells with 20 μCi of [3H]serine, 3 μCi of [3H]choline, or 2 μCi of [14C]ethanolamine per dish for various times, the culture medium was removed and saved. The cells were twice washed with 1 ml of cold PBS, and both washes were combined with the original culture medium. The cells were harvested by scraping, 3.5 ml of methanol was added, and the sample was vortexed. The sample was then incubated with 1 ml of cold acetone. The precipitated lipids were extracted from cell pellets and medium using a modified Folch procedure (35, 36). Radioactivity in lipid extracts was quantitated using a liquid scintillation counter. Phospholipids were separated using TLC with a solvent system of chloroform:ethanol:triethylamine:water (40:50:40:10,
v/v) and quantitated with a Bioscan 200 Imaging Analyzer. Total phospholipid mass was determined by measuring phosphorus content of the lipid extracts (37). Total phospholipid biosynthesis was normalized to total phosphorus in the lipid extracts. The data from three or four experiments were expressed as the means ± S.E., and the statistical differences were calculated using Student’s t test.

**Assay for PtdSer Externalization**—Cells grown on glass coverslips were rinsed with PBS. Annexin-V-fluorescein isothiocyanate and PI staining was performed according to the manufacturer’s instructions. After rinsing coverslips with binding buffer to remove unbound annexin-V and PI, the cells were fixed with 4% paraformaldehyde for 15 min and rinsed twice with PBS. The coverslips were mounted on glass slides. For the same area of each sample, a differential interference contrast image (for total cells), a green fluorescent image (for annexin-V-positive cells), and a red fluorescent image (for PI-positive cells) were acquired with the Zeiss LSM-510; superimposed images were obtained with LSM-510 Image software.

**Assay for PtdSer Synthase Activity**—After removal of the culture medium, the cell lysate was prepared by scraping cells in 0.5 ml of ice-cold suspension buffer (250 mM sucrose, 10 mM HEPES buffer, pH 7.5) and sonicating for 30 s on ice. The samples were centrifuged at 37°C for 40 min. The supernatant from cell extracts (100 µl) was added to an equal volume of prewarmed assay mixture (5 mM CaCl2, 50 mM HEPES buffer (pH 7.5), and 1 µCi of [3H]serine) and incubated at 37°C for 20 min (38). The reactions were terminated by adding 1.5 ml of methanol:H2O (5:4, v/v) and 2 ml of chloroform. The samples were mixed thoroughly and centrifuged at 9708 g for 10 min. Lipid was extracted using a modified Folch procedure, and the total radioactivity was determined using a liquid scintillation counter.

Protein concentration was measured using a Micro BCA kit.

**RESULTS**

**Generation and Characterization of CHO-K1 Cell Lines Stably Overexpressing Murine PLSCR1 or PLSCR2**—Previous studies indicate that endogenous PLSCR1 cannot be detected in CHO-K1 cells by Western blotting, and no scramblase activity is detected by in vitro enzyme assays (27). CHO-K1 cells were transfected with pcDNA-PLSCR1 or pcDNA-PLSCR2 constructs and clones stably overexpressing PLSCR1 and PLSCR2 were then selected. Initially, selection using G418 alone failed to produce positive clones, raising the possibility that cells overexpressing PLSCR were outgrown by cells where only the neomycin resistance gene was incorporated. We then used a strategy to select G418 and hygromycin B-resistant cells by co-transfecting cells with vector containing PLSCR and an empty vector containing a hygromycin resistance gene. With this approach, several G418/hygromycin-resistant clones were isolated. Introduction of a C-terminal Myc tag ensured that the overexpressed proteins could be detected by Western blotting and immunofluorescence using an anti-c-Myc mAb. A single protein band was detected in stable expressing clones transfected with pcDNA-PLSCR1 or PLSCR2 constructs, and both Myc-tagged-PLSCR isoforms migrated with predicted molecular weights. No endogenous protein from clones transfected with empty pcDNA3.1 vectors was recognized by anti-c-Myc mAb (Fig. 1A). Immunofluorescence analyses of these cells showed a predominantly plasma membrane localization of PLSCR1 cells (Fig. 1B, middle panel); in some cells, highly expressed PLSCR1 also was seen in the cell nucleus. Membrane vesiculation also was observed in cells overexpressing PLSCR1 (Fig. 1B, middle panel, arrow). Overexpressed PLSCR2 was found predominantly in the nucleus but also was apparent in the cytoplasm (Fig. 1B, right panel). Low background staining in vector control cells confirmed high specificity of anti-c-Myc mAb toward overexpressed PLSCR proteins (Fig. 1B, left panel). Collectively, our data indicated that CHO-K1 clones stably overexpressing c-Myc-tagged PLSCR1 and PLSCR2 were generated and that the levels of expression and subcellular localization of the introduced proteins could be readily detected with anti-c-Myc mAb. PLSCR-expressing clones with the best expression rate were used in the following experiments.

**Suppression of Cell Growth and Facilitation of UV-induced Apoptosis in CHO-K1 Cells by PLSCR1 Overexpression**—When cells were seeded at same density and monitored for 5 days for their growth rate, vector control cells doubled every 24 h similar to the wild type CHO-K1 cells, whereas cells overexpressing PLSCR grew at a slower rate; by day 4, cell growth was inhibited by 70% compared with vector control cells (Fig. 2A). The shape of PLSCR1-expressing cells was significantly different from that of wild type CHO-K1 cells, the former being rounder and smaller (Fig. 2B, left panels). PLSCR2-expressing cells were larger than vector control cells and had a similar growth rate from day 1–3. After reaching confluence (day 4 or 5), contact inhibition between PLSCR2-expressing cells resulted in a decreased growth rate compared with the vector control cells.

When apoptosis was induced in cells overexpressing PLSCR1 or PLSCR2 by exposing them to UV light for 10 min, cells overexpressing PLSCR1 developed much earlier signs of apoptotic morphology than UV-treated vector cells. Six hours after UV irradiation, when a majority of control cells with empty vector showed little signs of apoptosis, the PLSCR1-expressing cells became strongly light reflecting, and cell shrinkage was obvious (Fig. 2B, right panels). PLSCR2-expressing cells did not show major differences in developing apoptotic cell morphology compared with vector cells. When cell death was monitored with a trypan blue exclusion assay, PLSCR1-expressing cells showed a higher basal level of cell death, and UV treatment resulted in 30% dead cells, whereas no significant cell death was observed in UV treatment of vector cells 8 h after UV irradiation (Fig. 2C).

To detect pro-caspase-3 activation as a marker for the progression of apoptosis, antibody that only recognizes active caspase-3 was used to detect caspase activation in PLSCR1-expressing and vector cells (Fig. 3A). A higher basal level of cell death mediated by PLSCR1 overexpression was confirmed be-

![Fig. 1. Stable expression of c-Myc-tagged PLSCR1 or PLSCR2 in CHO-K1 cells. A, cells stably overexpressing PLSCR isoforms and controls transfected with empty pcDNA3.1 vectors were harvested, and the proteins were extracted and separated (20 µg) using SDS-PAGE and transferred to polyvinylidene difluoride membrane. c-Myc-tagged proteins were detected by Western blotting with anti-c-Myc mAb as described under “Experimental Procedures.” B, immunofluorescence was performed on CHO-K1 clones stably overexpressing PLSCR1 or PLSCR2 and on vector controls. The cells were fixed, permeabilized, and incubated with anti-c-Myc mAb. Alexa flour 488-conjugated goat anti-mouse IgG was used to detect Myc-tagged proteins. The cells expressing PLSCR1 cells were analyzed by confocal microscopy at a position highlighting the cell membrane. Membrane vesiculation was observed at the plasma membrane (arrows). The image of PLSCR2-expressing cells was taken at the midpoint of the nucleus. Bar, 10 µm. Vec, vector.
cause about 4% of the cells showed positive staining of active caspase-3 (Fig. 3B). Extensive activation of caspase-3 (65%) was observed in PLSCR1 cells, whereas only a few vector cells (15%) showed positive signals of activated caspase-3 following 8 h of incubation after UV irradiation. Nuclear condensation and fragmentation also were observed in apoptotic cells that showed positive staining for caspase-3 activation. No differences in caspase-3 activation and nuclear morphology were observed with PLSCR2-expressing cells compared with UV-treated vector cells. Thus, PLSCR1 overexpression conferred growth suppression and promoted UV-induced apoptosis in CHO-K1 cells, whereas PLSCR2 protein did not have similar effects.

*Increased PtdSer Exposure in PLSCR1-expressing Cells Induced with UV Light*—PLSCR1 is key in mobilizing phospholipids at the plasma membrane. Overexpression of PLSCR1 in CHO-K1 cells resulted in a slightly higher basal level of PtdSer externalization, whereas PtdSer on the cell surface remained low in vector control cells without UV irradiation (Fig. 4A). When apoptosis was induced with UV irradiation, PtdSer levels on the surface of PLSCR1 cells increased as early as 3 h after treatment (data not shown). By 8 h, the majority of PLSCR1 cells showed positive annexin-V-fluorescein isothiocyanate stain, indicating major PtdSer externalization, whereas UV-treated vector cells showed minor changes (Fig. 4). The integrity of cell membranes was maintained, and few cells were stained with propidium iodide in their nuclei. PLSCR2-expressing cells did not show major differences in exposure of PtdSer compared with vector cells with or without UV treatment (Fig. 4B). PtdSer externalization also was greatly inhibited by the presence of z-VAD-fmk both in UV-irradiated PLSCR1-expressing and vector cells (Fig. 4). Thus, PLSCR1 overexpression facilitated apoptosis and increased PtdSer externalization in CHO-K1 cells after UV irradiation in a caspase-dependent manner, whereas PLSCR2 overexpression did not appear to enhance PtdSer externalization or cell death.

*Stimulation of PtdSer Biosynthesis in PLSCR1-expressing Cells Following UV-induced Apoptosis*—Our previous study with U937 cells showed that de novo synthesis of PtdSer was stimulated 2–3-fold during programmed cell death induced by various stimulators of apoptosis (28). Our preliminary experiments indicated that similar stimulation of PtdSer biosynthesis also was observed in CHO-K1 cells during UV-induced apoptosis. To test the hypothesis that PtdSer biosynthesis and externalization to the cell surface were related events and that increased outward movement of PtdSer may further increase PtdSer biosynthesis, we measured the biosynthesis of PtdSer and other serine-derived phospholipids using cells overexpressing PLSCR1 or PLSCR2. After UV irradiation of cells followed by a 12-h incubation with [3H]serine, there was a 2-fold increase in PtdSer synthesis in vector cells compared with those without UV treatment (Fig. 5, top panel). PLSCR2-expressing cells had basal and UV-stimulated rates of PtdSer biosynthesis similar to control cells. Without any UV irradiation, the basal rate of PtdSer synthesis was slightly higher (1.7-fold) in cells overexpressing PLSCR1. With UV stimulation there was a significant increase in PtdSer biosynthesis in cells overexpressing PLSCR1; PtdSer biosynthesis was 4-fold higher compared with untreated PLSCR1-expressing cells. Although PtdSer de-carboxylation to PtdEtn slightly decreased following UV irradiation in vector and PLSCR2-expressing cells, de-carboxylation

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**Fig. 2.** Growth suppression and early occurrence of UV-induced apoptosis in CHO-K1 cells overexpressing PLSCR1. In A, CHO-K1 cells expressing PLSCR1, PLSCR2, and control clones were seeded at 1 × 10^5 cells/60-mm dish on day 0 and maintained in normal growth medium as described under “Experimental Procedures.” At the time indicated, the cell numbers were determined in triplicate samples and expressed as the means ± S.E. B and C show cells that were treated with or without UV light followed by 6 h (B) or 8 h (C) incubations. For B, phase contrast photographs were taken at 300× magnification. For C, cell death in PLSCR1-expressing cells and vector controls was assessed using a trypan blue exclusion assay as described under “Experimental Procedures.” The data are the means ± S.E. from triplicate samples of one representative experiment. Vec, vector.
was increased slightly in PLSCR1-expressing clones, indicating increased transport of newly synthesized PtdSer into the mitochondria (Fig. 5, middle panel). SM biosynthesis was stimulated 1.5-fold in UV-irradiated PLSCR1 or PLSCR2-expressing cells and 2.5-fold in vector cells following UV irradiation (Fig. 5, bottom panel). A higher amount of newly synthesized PtdSer also was recovered from medium (representing mainly vesicles released during apoptosis) with UV-treated PLSCR1-expressing cells compared with vector controls (Fig. 6).

In vitro serine base exchange assays were performed to test the possible involvement of altered PtdSer synthase levels in regulating PtdSer biosynthesis in cells overexpressing PLSCR1 (Table I). PLSCR1-expressing cells showed 30% higher serine base exchange activities compared with vector cells. UV treatment did not change the specific activities of PtdSer synthase.
enzymes in these cells. A 20% decrease was detected in UV-treated vector cells compared with untreated vector cells.

De novo biosynthesis of phosphatidylcholine monitored with radiolabeled choline was inhibited in PLSCR1-expressing cells by 25% following UV treatment, whereas no change was observed in UV-treated control cells. PtdEtn biosynthesis from radiolabeled ethanolamine was inhibited slightly in control cells and unchanged in PLSCR1 cells with UV treatment (Table II). Thus, overexpression of PLSCR1 cells resulted in a significant increase in PtdSer biosynthesis following UV-induced apoptosis, and newly synthesized PtdSer was released at a higher rate into medium as microvesicles.

Caspase Dependence of PtdSer Biosynthesis in PLSCR1-expressing Cells—Stimulation of PtdSer biosynthesis in wild type CHO-K1 cells during UV-induced apoptosis was independent of caspase activation and was not blocked by z-VAD-fmk, a general caspase inhibitor (data not shown). Cells overexpressing PLSCR1 showed significant basal levels of PARP cleavage, a marker for caspase-3 activation. When 100 μM z-VAD-fmk was added to cells after UV irradiation, apoptosis was blocked in both vector and PLSCR1-expressing cells, based on a lack of PARP cleavage (Fig. 7A). Stimulation of PtdSer biosynthesis was unchanged in the presence of z-VAD-fmk in UV-treated vector cells (similar to wild type CHO-K1 cells). PtdSer biosynthesis stimulated in PLSCR1-expressing cells was inhibited by z-VAD-fmk to a level similar to that of the UV-treated vector cells, indicating that further stimulation of PtdSer biosynthesis as a result of PLSCR1 overexpression was blocked by z-VAD-fmk (Fig. 7B). Movement of newly synthesized PtdSer into microvesicles was sensitive to z-VAD-fmk in both vector and
PLSCR1-expressing cells (Fig. 7C). z-VAD-fmk showed minimal effects on PtdSer decarboxylation in vector and PLSCR1-expressing cells treated with or without UV irradiation. UV-stimulated SM synthesis in vector cells was not changed by z-VAD-fmk, but the caspase inhibitor further increased SM stimulation in UV-treated PLSCR1-expressing cells to the level similar to that of the UV-treated vector cells (data not shown). Thus, stimulation of PtdSer biosynthesis in PLSCR1 cells was partially dependent on caspase activation, but caspase-independent mechanisms were involved in up-regulating PtdSer biosynthesis in vector cells after induction of apoptosis by UV exposure.

**DISCUSSION**

Externalization of PtdSer on the cell surface is a key signal for removal of apoptotic cells. Our previous studies showed that transbilayer migration of PtdSer was correlated with PtdSer synthesis by a caspase-dependent process (28). To test the postulated relationship between PtdSer externalization and its biosynthesis, we studied the involvement of PLSCR isoforms responsible for active translocation of PtdSer to the cell surface in regulating PtdSer biosynthetic pathways. Stable overexpression clones of two PLSCR isoforms were established in CHO-K1 cells. Because endogenous PLSCR1 cannot be detected in CHO-K1 cells by Western blotting and Because no scramblase activity is detected by in vitro assays (27), we anticipated that addition of PLSCR to the cells by transfection might enhance PtdSer synthesis and/or its externalization in response to induction of apoptosis.

When overexpressed in CHO-K1 cells, PLSCR1 was located predominantly in the plasma membrane of the cell and, in some cases, in the nuclei. Palmitoylation at conserved cysteines in PLSCR1 is required for anchoring this protein to the plasma membrane (26); overexpressed PLSCR1 proteins not modified by palmitoylation may be moved into the nuclei. Overexpression of PLSCR1 resulted in changes in cell morphology, increased basal cell death, and slower rate of cell growth. Noticeably, PLSCR1-expressing cells also had higher basal levels of PtdSer expression on the cell surface. Membrane blebbing was observed in these cells. When PLSCR1 is stably expressed in ovarian carcinoma cell line HEY1B (20), no changes in growth rate and morphology are found in cells grown in serum culture, but significant suppression of tumor development is observed when PLSCR1-expressing cells are implanted into athymic nude mice (20). Our data indicate that PLSCR1 can suppress cell growth in serum culture under different cell contexts. The implications or mechanisms of morphological changes in PLSCR1 overexpressing cells are not clear, but increased membrane lipid movement and blebbing may contribute to the observed changes. PtdSer is found to be preferentially exposed in membrane domains, particularly in membrane blebs (39), indicating that higher PLSCR1 activity may direct PtdSer to these surface regions.

When apoptosis was induced by UV irradiation, PLSCR1-expressing cells developed morphological and biochemical changes much earlier than the UV-treated vector cells. First, PLSCR1 cells rapidly exposed PtdSer to the surface; by 8 h
following UV stimulation, majority of the cells overexpressing PLSCR1 showed positive annexin-V binding, whereas very few control cells had exposed PtdSer. Second, caspase-3 activation occurred much earlier in PLSCR1 cells compared with controls. Third, nuclear fragmentation in PLSCR1 cells preceded that in control cells. Collectively, these dramatic differences indicated that PLSCR1 plays an important role in promoting PtdSer externalization following induction of apoptosis. Thus, PLSCR1 can be considered to be anti-proliferative and pro-apoptotic as overexpression leads to suppression of growth and extensive cell death in untreated cells and facilitates apoptosis in UV-induced cells.

To determine relationships between increased PtdSer movement to the outer surface and synthesis of new PtdSer, lipid biosynthesis was examined using labeled serine as a precursor. Based on incorporation and metabolism of labeled serine by intact cells, clones overexpressing PLSCR1 showed higher basal levels of PtdSer biosynthesis and externalization compared with vector control cells. In vitro assays of serine base exchange activity (recognizing that maximal activities measured under in vitro conditions may not fully reflect the capacity for synthesis in intact cells within a localized milieu of in vivo activators or inhibitors) suggest that an increase in PSS enzyme activity in PLSCR1-expressing cells may contribute to the higher basal levels of PtdSer biosynthesis measured in intact cells.

Following UV irradiation of intact cells, the stimulation of PtdSer biosynthesis measured by labeled serine incorporation was significantly higher in PLSCR1-expressing cells compared with control cells containing empty vector, and more newly synthesized PtdSer moved into microvesicles; apoptotic cells overexpressing PLSCR1 had a 2.5-fold increase in PtdSer synthesis compared with UV-treated vector cells. Potentially, an increase in PtdSer level could be explained by a blockage of PtdSer decarboxylation, but this apparently was not the case, because synthesis of serine-derived PtdEtn was even slightly enhanced in PLSCR1-expressing cells following UV irradiation, indicating increased transport of PtdSer into mitochondria. Also, no major changes in serine uptake were observed in PLSCR1-expressing cells and vector cells following UV irradiation (data not shown). Although not a predicted correlation, specific activities of serine base exchange enzymes measured in vitro remained unchanged in apoptotic PLSCR1-expressing cells upon UV treatment, indicating that an increase in the cellular content of base exchange enzymes may not be the primary reason underlying the stimulation of PtdSer biosynthesis observed in both cell lines. This implies that changes in cellular PtdSer biosynthesis during triggering of apoptosis involves more than an increase in the amount of available enzyme. As one explanation of this unanticipated observation, we propose that activation of PtdSer externalization to the cell surface of intact cells, facilitated through overexpression of PLSCR1, may relieve PtdSer feedback to enhance base exchange activity and new PtdSer biosynthesis in both untreated and UV-treated PLSCR1-expressing cells without a change in actual amount of PSS enzyme. Movement of PtdSer to the outer leaflet of the plasma membrane and its migration into vesicles may deplete PtdSer on the inner surface of the membrane bilayer. This may produce a signal for enhanced PtdSer biosynthesis as PtdSer-mediated inhibition of the production of new PtdSer is released (Fig. 8).

In contrast to the greater effect on PtdSer synthesis, PLSCR1-expressing cells had only a 1.5-fold increase in SM formation compared with treated control cells and a 2.5-fold stimulation following UV irradiation. It seems that pathways of PtdSer biosynthesis and trafficking are up-regulated to a greater extent in cells overexpressing PLSCR1 even though the SM biosynthesis pathway shares serine as a common substrate.

In both the parent CHO-K1 cells and vector-transfected controls, the 2-fold stimulation of PtdSer synthesis following UV irradiation occurred independently of caspase activities. Other studies in our laboratory indicate that UV-mediated stimulation of PtdSer synthesis in CHO-K1 cells, catalyzed by overexpressed PSS I or PSS II, does not require caspase activation. On the other hand, the 4-fold stimulation of PtdSer formation in PLSCR1-expressing cells seems to have both caspase-dependent and caspase-independent components as co-incubation of z-VAD-fmk reduced PtdSer levels in UV-treated PLSCR1-expressing cells back to that of the UV-treated control cells but did not reduce it to the level of untreated cells. The caspase-regulated step appears to be PtdSer externalization mediated by PLSCR1. The serine base exchange reaction per se does not require activation of caspases; it only becomes sensitive to caspase inhibition when its activity is triggered by PtdSer externalization, possibly through indirect release of feedback inhibition (Fig. 8).

When PLSCR2, a shorter isoform of scramblase without an extracellular tail was expressed, the protein was found predominantly inside the nucleus. PLSCR2 overexpression does not facilitate PtdSer externalization or alter PtdSer biosynthesis in CHO-K1 cells during UV-induced apoptosis. This further confirms that PLSCR1-mediated PtdSer externalization leads to the stimulation of PtdSer formation. Mouse PLSCR2 when reconstituted into proteoliposomes in vitro has been reported to catalyze NBD-PC scrambling similar to human PLSCR (21). Possibly, PLSCR2 was not targeted to the right region of our cells, and its presence in the nucleus is inadequate to influence PtdSer externalization and apoptosis.

In summary, our data indicate that PLSCR1, when overexpressed in CHO-K1 cells, promotes externalization of PtdSer at the plasma membrane and facilitates UV-induced apoptosis. The PLSCR2 isoform is targeted primarily to the nucleus where its role is not clear. PtdSer biosynthesis is greatly stimulated in PLSCR1-overexpressing cells induced to undergo apoptosis. This stimulation is dependent on caspase activation, possibly mediated by feedback by altered levels of PtdSer on the inner surface of the plasma membrane to increase base exchange activity without changing levels of the PSS enzyme. Thus, PLSCR1 at the plasma membrane appears to be a point of control in regulating the apoptotic process and may mediate a feedback signal that results in enhanced biosynthesis to replace mobilized PtdSer. Continued PtdSer synthesis may be required for sustained apoptosis. It is apparent that the externalization of PtdSer mediated by altered scramblase activities is a potential point of regulation and hence a possible target for therapeutic intervention in the complex process of programmed cell death and removal of dead cells and debris from the body by immune response systems.

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Stimulation of Phosphatidylserine Biosynthesis and Facilitation of UV-induced Apoptosis in Chinese Hamster Ovary Cells Overexpressing Phospholipid Scramblase

Anan Yu, Christopher R. McMaster, David M. Byers, Neale D. Ridgway and Harold W. Cook

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