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METABOLISM AND EXTERNALIZATION OF PHOSPHATIDYLSERINE DURING APOPTOSIS IN CULTURED CELLS WITH AND WITHOUT OVER-EXPRESSION OF METABOLIC ENZYMES

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Dalhousie University

at

Department of Biochemistry & Molecular Biology
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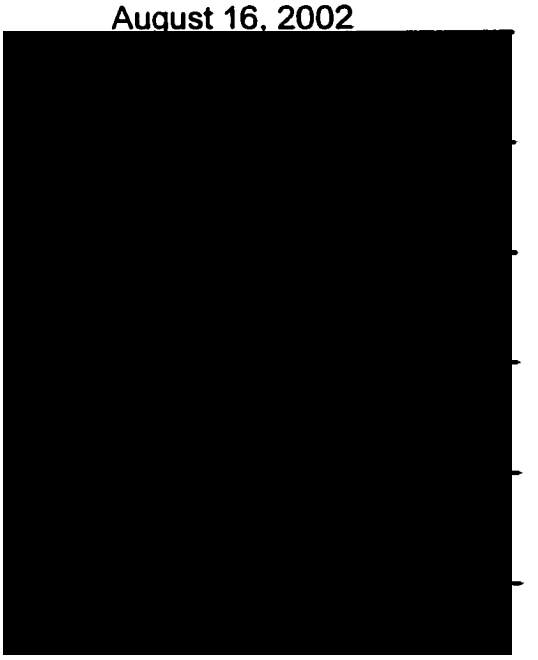

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Signature of Author

For Grandpa

For Mom and Dad

For my dear sisters

For Pengpeng and Chengcheng

It is great to have you as my family.

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Abstract

Externalization of phosphatidylserine (PtdSer) plays important roles in signaling the recognition and removal of apoptotic cells. We hypothesized that PtdSer exposed during apoptosis is newly formed and that biosynthesis of PtdSer correlates with its externalization. In this study, U937 and CHO-K1 cells were induced with various apoptotic stimuli and metabolism of serine-derived phospholipids was studied. In U937 cells, PtdSer synthesis was stimulated and newly synthesized PtdSer was transferred preferentially to apoptotic bodies when apoptosis was induced with camptothecin. Stimulation of PtdSer synthesis and transport to apoptotic bodies were abolished by a caspase inhibitor, z-VAD-fmk. In contrast, changes in synthesis and transport of other phospholipids were minor. Even greater effects on PtdSer synthesis, movement to vesicles and inhibition by z-VAD-fmk were observed in apoptotic cells induced by UV irradiation or tumor necrosis factor-α. In CHO-K1 cells UV-induced apoptosis resulted in a 2-fold increase in PtdSer biosynthesis but this was not reversed by z-VAD-fmk and was less specific for PtdSer as similar levels of stimulation were observed for sphingomyelin biosynthesis. Thus, increased PtdSer biosynthesis may be a general phenomenon during apoptosis but the stimulation of PtdSer synthesis may involve distinct regulatory pathways depending on the type of cell involved. In U937 cells, this event is specific for PtdSer and depends on caspase activation whereas in CHO-K1 cells, stimulation of PtdSer synthesis seems to be less specific and independent of caspase activities.

PtdSer formation in mammalian cells is catalyzed by PtdSer synthases (PSS) that convert existing phospholipids to PtdSer. Members of the phospholipid scramblase (PLSCR) family contribute to exposure of PtdSer in the outer leaflet of the plasma membrane during apoptosis. CHO-K1 cells over-expressing isoforms of PSS or PLSCR were established to determine whether PtdSer biosynthesis associated with apoptosis is altered by their activities. PSS I- and PSS II-expressing cells showed resistance to UV-induced apoptosis based on a lack of caspase-3 activation or morphology and nuclear changes. When exposed to UV light, PtdSer biosynthesis was further stimulated approximately 2-fold in PSS I cells and 3-fold in PSS II cells compared to treated control cells. Caspase activation was not required as z-VAD-fmk did not change PtdSer metabolism in PSS I or PSS II-expressing cells. Cells over-expressing PLSCR1 showed significant morphological changes and cell death. Following UV irradiation, these cells had earlier and enhanced PtdSer exposure, and increased caspase-3 activation, PARP cleavage and nuclear changes. UV-irradiated cells expressing PLSCR1 had a 6-fold stimulation of PtdSer synthesis relative to untreated PLSCR1 cells whereas UV-irradiated vector control cells increased only 2-fold. No differences in these responses were observed in PLSCR2-expressing cells. PtdSer synthesis and apoptosis stimulated by PLSCR1 over-expression were blocked by z-VAD-fmk.

In conclusion, PSS I and II appear to be involved in the formation of PtdSer following UV irradiation through caspase-independent mechanisms. Cells over-expressing PSS I and II are resistant to UV-induced apoptosis, indicating potential anti-apoptotic effects when these synthetic enzymes are over-expressed. Over-expression of PLSCR1 in CHO cells increased PtdSer synthesis and externalization and enhanced the rate of apoptosis in a caspase-dependent manner following UV irradiation. Thus, stimulation of PtdSer biosynthesis seems to be directly related to PtdSer externalization and this could be a point of regulation or intervention during programmed cell death.

List of Abbreviations

ABC	ATP-binding cassette
APT	aminophospholipid translocase
bp	base pair
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CHX	cycloheximide
dH_2O	distilled water
DMSO	dimethylsulfoxide
DMEM	Dulbecco's modified Eagle's medium
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
E.coli	*Escherichia coli*
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein-5-isothiocyanate
HEPES	*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HRP	horseradish peroxidase
kDa	kilodalton
LB	Luria-Bertani medium
MAM	mitochondria-associated membranes
MARCKS	myristoylated alanine-rich C kinase substrate
MDR	multidrug resistance

MRP1	multidrug resistance (-associated) protein 1
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PSR	phosphatidylserine receptor
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdSer	phosphatidylserine
PCR	polymerase chain reaction
PI	propidium iodide
PM	plasma membrane
PSS I	phosphatidylserine synthase I
PSS II	phosphatidylserine synthase II
PLSCR	phospholipid scramblase
PKC	protein kinase C
PVDF	polyvinylidene difluoride
RBCs	red blood cells
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SLE	systemic lupus erythematosus

SM	sphingomyelin
SR-BI	class B scavenger receptor type I
STS	staurosporine
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor α
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
z-VAD-fmk	N-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethyl ketone

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I worship the memory of my late grandfather.

Finally, I would like to thank my parents for raising me and providing me a loving environment to grow up and for their never-ending love and support for me.

I. Introduction

A. Programmed cell death

1. Discovery and definition of apoptosis

a. Historical perspective

Over the last decades, great enthusiasm has developed toward improving our understanding of the process of physiological cell death, a natural phenomenon called apoptosis that is now considered to be as crucial as cell proliferation in regulating the homeostasis of living organisms. In one of the first documents recognizing cell death, the famous German anatomist, Walther Flemming, published a drawing of cells observed during normal regression of ovarian follicles. These depictions in 1885 showed great resemblance to what is now well characterized as apoptosis. At that time, the process was called "chromatolysis". Ludwig Gräpper, another German anatomist with great foresight, predicted in 1914 that chromatolysis may be a mechanism that exists to counterbalance mitosis (Majno and Joris, 1995; Kerr, 1999). Both of these important observations were ignored for several decades as cell death was extremely difficult to either observe or record due to the limitations of microscopic technology.

A significant breakthrough came with the histochemical studies that examined the lysosomal changes of cells in hepatic ischemia (Kerr, 1965). These experiments identified that shrinkage of ischemic lobes was due to the conversion of affected cells into small round cytoplasmic masses followed by their phagocytosis by Kupffer cells and epithelial cells. These cytoplasmic masses contain condensed nuclear chromatin, and intact organelles such as lysosomes, ribosomes and mitochondria. The term "shrinkage necrosis" was used to distinguish this form of cell death from necrosis (Kerr, 1971).

Shrinkage necrosis also was observed in rat tissues treated with toxic agents (Kerr, 1969), in tumors (Kerr and Searle, 1972), in adrenal cortices where hormone secretion was interrupted (Kerr, 1972) and in normal embryonic and fetal development (Crawford et al., 1972). A new term, apoptosis, was adapted to describe this widespread and novel form of cell death based on the Greek word for falling of leaves from trees (Kerr et al., 1972).

b. Definition of cell death

Several terms are now used to describe cell death. Apoptosis defines a form of cell death with distinct morphological and biochemical characteristics. Programmed cell death emphasizes that all information controlling the demise of a cell comes from its own genes as if the cell is programmed to die from the moment it is created. Controlled physiological cell death is the way organisms remove unwanted individual cells to achieve overall homeostasis under normal physiological conditions. Apoptosis occurs relatively rapidly with dead cells being cleared without provoking inflammatory reactions (Fadok and Henson, 1998). In contrast, necrosis, also known as accidental cell death, defines a more explosive form of death whereby damage suffered by cells under extreme conditions leads to the complete rupture of all membrane compartments and the release of cellular contents to initiate inflammation (Trump et al., 1997).

2. Characteristics and detection of apoptosis

Studies of apoptosis are complicated by the existence of multiple forms of cell death. The decision of whether a cell will undergo apoptosis or necrosis appears to depend on the harshness of the death stimuli. To facilitate the studies of these processes, it is crucial to be able to detect specifically the induction of apoptosis through distinct morphological and biochemical characteristics that are different from those of necrosis. Apoptosis is a

highly asynchronous and dynamic process which means that cells, even within the same population, respond to death stimuli with changes appearing at different phases of this process. Further, some markers of the process may be unstable and may change depending on the stage of progression of apoptosis. Most studies have used cultured cells but increasingly more studies use whole animal models. Accordingly, for appropriate interpretation of apoptosis studies, it is important to understand the merits and limitations of techniques used to analyze the progression of apoptotic processes (Mesner, Jr. and Kaufmann, 1997; Hall, 1999; Willingham, 1999).

a. Morphological changes during apoptosis

(1) General cell morphology

Apoptotic cells share common features of morphology that can be detected using microscopic approaches ranging from routine phase contrast or fluorescence microscopy to more sophisticated differential interference contrast (DIC) microscopy or video time-lapse microscopy to assess the kinetics of apoptosis of cells in culture. For most adherent cells, morphological changes associated with apoptosis start with detachment and subsequent release of cells from the substratum resulting in round shaped cells. Massive membrane blebs then develop on the surface followed by shrinkage of cells and formation of apoptotic bodies. Some cells form elongated spikes on the surface. In contrast, necrotic cells usually swell, rather than shrink, and lose membrane integrity so that the whole cells break with the release of pathogenic contents (Hacker, 2000).

Apoptotic cells progressively assemble intact organelles and fragmented nuclei into apoptotic bodies for clearance. During the final stage of apoptosis, cells develop blisters on the surface and finally lyse in a manner similar to necrotic cells. In living organisms,

apoptotic cells are rapidly removed by surrounding cells and rarely last to the late lysing stage (Willingham, 1999; Webb et al., 1997). Organelles such as endoplasmic reticulum (ER), Golgi apparatus and lysosomes remain intact in the process of apoptosis whereas the outer membrane of mitochondria ruptures releasing proteins that are crucial in the initiation of apoptosis (Strasser et al., 2000).

(2) Nuclear segregation

Significant morphological changes inside the nuclei of apoptotic cells can be detected with cell-permeant nuclear dyes that stain the minor groove of dsDNA, such as DAPI (4',6'-diamidino-2-phenylindole) or Hoechst dyes, or with dyes, such as propidium iodide (PI), that are permeable only upon disruption of plasma membrane integrity and stain by intercalating into nucleic acid molecules (Mesner, Jr. and Kaufmann, 1997; Nicoletti et al., 1991). Apoptotic changes in the nucleus start with the condensation and margination of chromatin along the nuclear membrane. Nuclei then segregate into small, compact fragments with homogenous chromatin masses (Martelli et al., 2001).

b. Biochemical changes during apoptosis

(1) Cell surface changes

One of the hallmarks of apoptosis is the transbilayer redistribution of aminophospholipids, especially phosphatidylserine (PtdSer), at the cell surface. PtdSer is normally confined to the inner layer of the plasma membrane but externalization of PtdSer to the outer leaflet occurs relatively early in the process of apoptosis (Martin et al., 1995a). Externalization of PtdSer can be detected using fluorescently labeled annexin-V, a protein that binds to PtdSer with high affinity in the presence of Ca^{2+} (Reutelingsperger and van Heerde, 1997; Tait and Gibson, 1992). Membrane integrity is essential in

detecting exposed PtdSer because annexin-V will enter the cell if the membrane is leaky and stain PtdSer in the inner leaflet of the plasma membrane. Thus, it is instructive also to use propidium iodide, a membrane-impermeant nuclear dye as an indicator of plasma membrane integrity (van Engeland et al., 1998; van Engeland et al., 1996).

(2) DNA degradation

A variety of endonucleases activated during apoptosis break the DNA chains between the linker regions of nucleosomes to produce DNA fragments that are multiples of 150~200 bp in length (Walker and Sikorska, 1997). These fragments will produce a pattern known as "DNA ladders" in agarose gel electrophoresis. Individual apoptotic cells can be detected by labeling nicked or single stranded DNAs. The so called TUNEL (TdT-mediated dUTP nick end labeling) assay is an effective approach whereby terminal deoxylnucleotidyl transferase catalyzes the incorporation of fluorescently labeled dUTP into nicked DNA ends to produce signals for visual and quantitative detection (Heatwole, 1999).

(3) Proteolysis of cellular substrates

Apoptosis is executed by a variety of proteases (Martin and Green, 1995; Kidd et al., 2000). Many cellular proteins are substrates for proteolytic cleavage and can be used as markers for detection of apoptotic cells. Caspase proenzymes, poly(ADP)ribose polymerase (PARP) and fodrin are frequently used in immunoblotting detection of the formation of cleaved fragments after proteolysis (Tewari et al., 1995; Martin et al., 1995b).

3. Molecular mechanisms of apoptosis

Apoptosis is a complex cellular event including an initiation phase and an execution phase. A variety of external and internal stimuli signal cells for self-destruction. Due to the diversity of death signals, the initiation phase of apoptosis involves several different signaling pathways as well as regulatory mechanisms. However, all pathways appear to converge on a more restricted execution phase so that a common death machinery is activated; as a result, common cellular and biochemical changes are produced in apoptosis induced by different stimuli (Strasser et al., 2000).

Many types of stress inflicted on a cell may result in triggering of programmed cell death. Under pathological conditions, these stresses include hypoxia, hyperthermia, hypoglycemia, death ligand interaction, DNA damage by genotoxin or irradiation, withdrawal of growth factors or infections by pathogens (Leist and Nicotera, 1997).

a. Death receptors and adaptor proteins

Members of tumor necrosis factor (TNF) family are among the best characterized death initiators. These initiators are located on the plasma membrane or are secreted by other cells to induce apoptosis of target cells. TNFs initiate signaling through membrane receptors belonging to tumor necrosis factor receptor (TNFR) family. The TNFR family is characterized by an extracellular cysteine-rich domain and an intracellular death domain (DD). Signals from membrane receptors are relayed to downstream adaptor proteins through homotypic interactions between DDs on both proteins (Fig. 1). In addition to multiple DD domains, these adaptors have death effector domains (DED) that are responsible for recruiting downstream proteins, such as DED-containing procaspase-8 (section I.A.3.d). This protein complex is called DISC (death-inducing signaling

Figure 1. An example of death-domain-mediated apoptosis through activation of caspase-8. Members of the tumor necrosis factor (TNF) family, such as TNF-α, trigger apoptosis by initiating the trimerization of TNF receptors (TNF-R1) on the plasma membrane. Homotypic interaction occurs between death domain (DD) of TNF-R1 with the DD regions of adaptor proteins including TRADD (TNF receptor-associated death domain protein) and RIP (receptor-interacting protein). TRADD recruits and activates downstream pro-caspase-8 containing a DD region, resulting in the maturation of effector caspases such as caspase-3 and the execution of the death program. This apoptotic signaling pathway competes with a survival signaling pathway mediated by the phosphorylation inactivation of inhibitors of NF-κB (I-κB). The illustration is adapted from a similar one (Ashkenazi and Dixit, 1998).

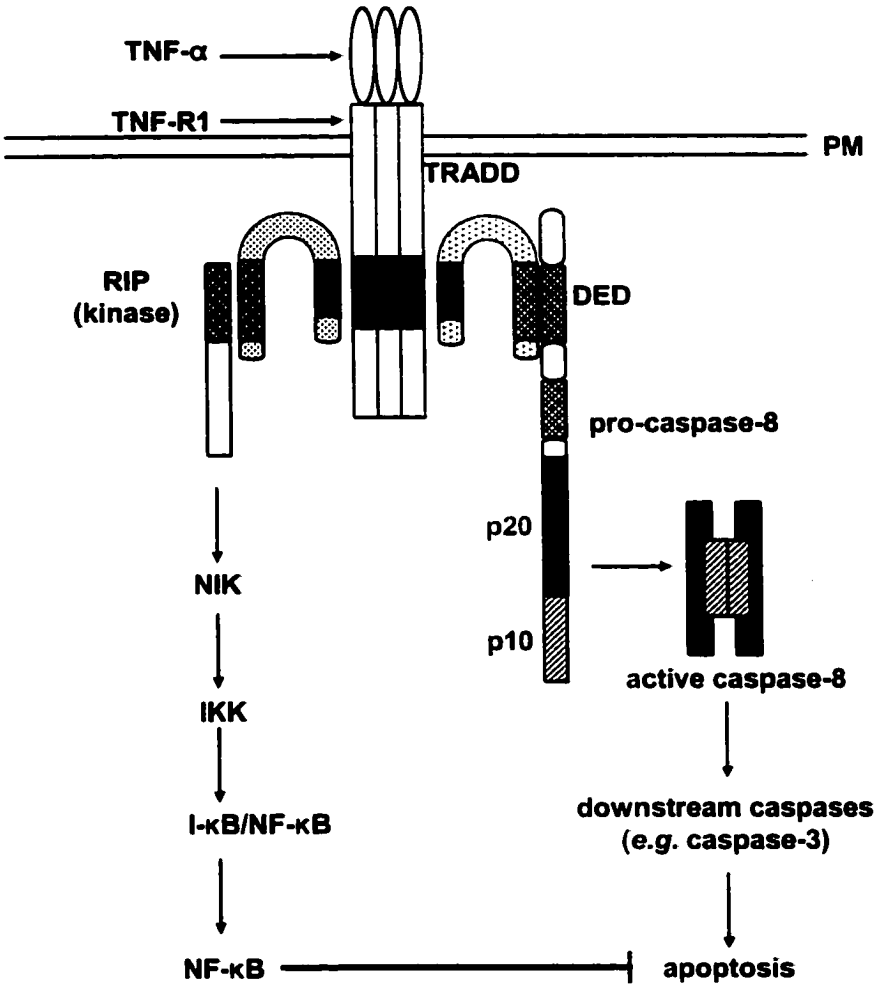
TNF-α
TNF-R1
PM
TRADD
RIP
(kinase)
DED
pro-caspase-8
p20
p10
active caspase-8
NIK
IKK
I-κB/NF-κB
downstream caspases
(e.g. caspase-3)
NF-κB
apoptosis

Figure 1

complex). The resulting high local concentrations of procaspase-8 lead to the autocatalytic proteolysis and assembly of active caspase-8 to initiate apoptosis (Wallach et al., 1999; Ashkenazi and Dixit, 1998; Nagata, 1997; Weber and Vincenz, 2001).

b. DNA damage and p53

DNA damage induced by UV or γ-irradiation or other genotoxic agents is one of the most effective ways whereby apoptosis is induced in most cells. The integrity of genomic DNA is vital for the healthy survival of mammalian cells. DNA damage must be repaired before subsequent DNA replication and compaction into chromosomes or otherwise permanent mutations will occur. Thus, cells initially respond to DNA damage through cell-cycle arrest which provides time for DNA repair. When DNA damage is too severe, apoptosis is initiated to eliminate the potentially dangerous cells (Fig. 2) (Norbury and Hickson, 2001).

p53, a transcription factor known for its tumor suppression role, is a key mediator in apoptosis induced by DNA damage (Steele et al., 1998; Burns and El Deiry, 1999). Like other transcription factors, p53 has a transactivation domain and a DNA binding domain. Under normal conditions p53 has a short half-life because MDM2 protein binds to the transactivation domain of p53 leading to its degradation (Lane and Hall, 1997). p53 activity is further inhibited by interaction between its C-terminal region and the DNA binding domain to prevent accidental activation of p53 activity. When cells sense DNA damage, several serine protein kinases will be activated and phosphorylate key serine residues in the transactivation domain of p53. This phosphorylation will block MDM2 binding and stabilize p53 (Salomoni and Pandolfi, 2002). Acetylation and dephophorylation on the C-terminus of p53 also relieve the inhibition of DNA-binding

Figure 2. An example of signaling pathways of UV-induced apoptosis. UV irradiation causes DNA damage and direct trimerization of TNFR on the cell surface and thus initiates two main apoptotic signaling pathways. Cells sense DNA damage through activation of protein kinases that further mediate stabilization and activation of p53, a transcriptional factor. As a result, expression of pro-apoptotic proteins, such as the Bcl-2 family or TNF/TNFR family members, is up-regulated whereas expression of anti-apoptotic proteins is down-regulated. Mitochondria-associated apoptosis is initiated by higher levels of pro-apoptotic members of the Bcl-2 family. This leads to formation of apoptosomes and activation of caspase-9. p53 may also increase the expression of members of TNF and TNFR family and trigger apoptosis through the activation of caspase-8. The illustration is adapted from a similar one (Burns and El Deiry, 1999).

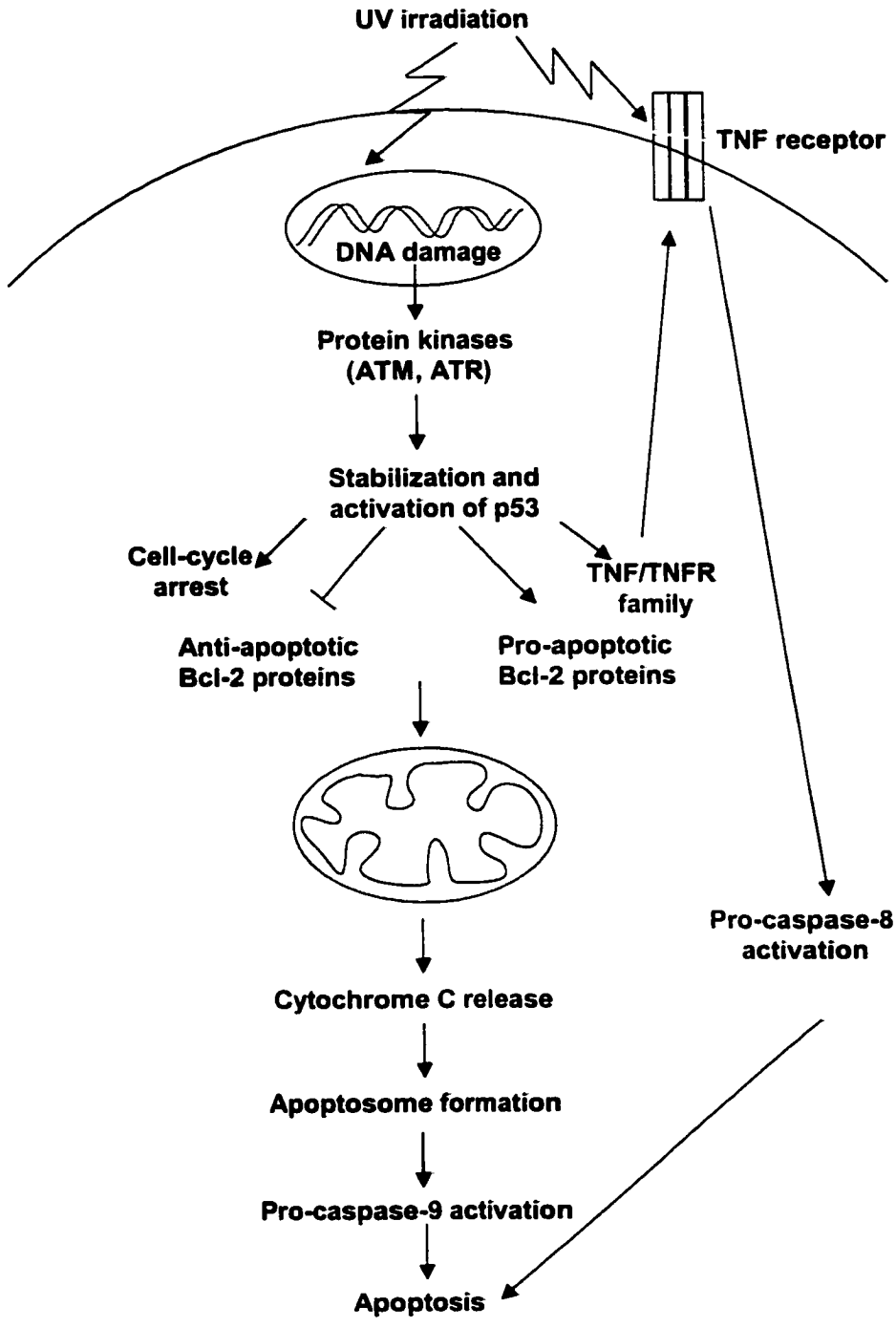
UV irradiation
TNF receptor
DNA damage
Protein kinases
(ATM, ATR)
Stabilization and
activation of p53
Cell-cycle
arrest
TNF/TNFR
family
Anti-apoptotic
Bcl-2 proteins
Pro-apoptotic
Bcl-2 proteins
Pro-caspase-8
activation
Cytochrome C release
Apoptosome formation
Pro-caspase-9 activation
Apoptosis

Figure 2

ability. Transcription of p53-induced genes is activated by p53 to regulate G1 or G2 cell-cycle arrest or apoptosis. For example, expression of key regulators in initiating apoptotic signaling pathways, such as death receptors (Fas and TNFR) and pro-apoptotic Bcl-2 family, is up-regulated by p53. Furthermore, expression of the anti-apoptotic Bcl-2 family is down-regulated by p53 activity to ensure induction of apoptosis. Bcl-2 is a potent inhibitor of p53 activation indicating the existence of a complete feedback control loop (Rich et al., 2000; Sheikh and Fornace, Jr., 2000a).

c. Bcl-2 family and mitochondria-mediated apoptosis

A death pathway initiated through mitochondria in response to DNA damage or other stresses operates in parallel to receptor-mediated apoptosis (Strasser et al., 2000). The regulators involved are pro- and anti-apoptotic members of the Bcl-2 family. Bcl-2 is identified as an inhibitor of apoptosis in B cell lymphocytes and is the mammalian homologue of the CED-9 protein of *C. elegans*. Bcl-2 protein contains four Bcl-2 homology (BH) regions, known as BH1-4; other members of the family have at least one BH region similar to Bcl-2. The Bcl-2 family is divided into three subgroups based on the number of BH regions and their roles in regulating apoptosis; anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-x_L, contain 3-4 BH regions and inhibit induction of apoptosis by maintaining mitochondrial homeostasis and membrane integrity. Pro-apoptotic Bcl-2 proteins including Bax and Bak, have 2-3 BH regions and promote apoptosis by mediating the release of cytochrome C and other proteins from mitochondria. These two subgroups also contain a transmembrane domain and localize to the outer mitochondrial membrane. Members of a third subgroup, including Bad, Bim and Bid, are soluble in the cytosol and contain only one BH3 region. They have high

potency in promoting apoptosis and in regulating the activities of the other two membrane-associated subgroups by relaying death signals from the cytosol to mitochondria (Adams and Cory, 2001).

Pro-apoptotic and anti-apoptotic members of Bcl-2 family interact with each other. Thus, the molecular ratio between these two subgroups plays a key role in the induction of apoptosis; higher levels of pro-apoptotic members lead to cell death whereas higher concentrations of the anti-apoptotic subgroups favor survival (Reed, 1997). Activation of p53 will up-regulate the expression of pro-apoptotic Bcl-2 proteins and the BH3-only subfamily while simultaneously decreasing expression of anti-apoptotic Bcl-2 subgroups to move the balance towards apoptosis. Furthermore, BH3-only members, when activated in cytosol by upstream death signals, will translocate to mitochondria to block the inhibitory effects of anti-apoptotic members. Bid translocation is induced by cleavage by proteases such as caspase-8, 3 and 7 (section I.A.3.d). Bad is phosphorylated by serine kinases such as Akt under normal conditions, and thus is able to bind 14-3-3 protein and remain in the cytosol. Dephosphorylation of Bad in the absence of survival signals leads to translocation to the outer mitochondrial membrane and blockage of the function of anti-apoptotic Bcl-2 proteins (Wang, 2001).

The pro-apoptotic Bcl-2 family induces apoptosis by releasing several proteins from the mitochondria to the cytosol. Many theories have been proposed to explain how this release is regulated. In general, rupture of the outer mitochondria membrane or formation of pores large enough for protein passage is believed to be controlled by the Bcl-2 family through unidentified mechanisms. Cytochrome C, apoptosis inducing factor (AIF),

Smac/DIABLO, and endonuclease G (EndoG) are among the proteins released (Hengartner, 2000; Bernardi et al., 2001).

Cytochrome C, the only soluble component of the mitochondrial electron transfer chain, is located in the inner space between two mitochondria membranes. Release of cytochrome C into the cytosol initiates formation of an apoptosome, a multiple protein complex similar to the DISC complex (Adrain and Martin, 2001). Apaf-1 (apoptosis activation factor) is a cytosolic adaptor protein that is also involved and contains a CARD (caspase recruitment domain) region, a nucleotide binding domain and multiple WD-40 repeats. Apaf-1 by itself cannot bind ATP with high affinity and thus exists as a monomer. When cytochrome C binds to Apaf-1, it increases the association of Apaf-1 with ATP through a conformational change. ATP binding induces oligomerization of Apaf-1 and cytochrome C to form apoptosomes and exposes the CARD region of Apaf-1. As a result, downstream CARD-containing caspases (section I.A.3.d), such as procaspase-9, are recruited to apoptosomes and activated (Bratton and Cohen, 2001; Strasser et al., 2000; Green and Reed, 1998).

d. Family of caspases

A family of cysteine-activated aspartate-specific proteases, known as caspases, lies at the center of the cascade of intracellular events supporting apoptosis (Wolf and Green, 1999; Salvesen and Dixit, 1997). Based on catalytic sites consisting of a highly conserved cysteine residue, caspases catalyze the cleavage of peptide bonds at the carboxyl terminal of aspartic acid (P1 position). The substrate specificity of each caspase is determined by the third amino acid (P4 position) located upstream of the aspartate at the P1 position. Caspases are produced as inactive zymogens to avoid inadvertent induction of apoptosis.

Two kinds of proteases catalyze the maturation of procaspase: one consists of members of the caspase family themselves and the other is granzyme B formed by cytotoxic T lymphocytes. Cleavage at sites on the carboxyl terminal aspartates of procaspases results in the formation of small and large units that interact. Two such heterodimers then interact through their small units to form active caspase, a heterotetramer comprised of two large and two small subunits (Cohen, 1997; Thornberry and Lazebnik, 1998).

The fourteen caspase homologues so far identified in mammals can be divided into two groups based on their functions in regulating cell death (Earnshaw et al., 1999). The initiator caspases, characterized by long prodomains, relay the death message from upstream adaptor proteins to downstream caspases and mediate their activation. The effector caspases, after being activated by initiator caspases, are responsible for the cleavage of multiple protein substrates leading to apoptotic morphology and biochemical changes. All caspases contain large and small catalytic subunits linked by one or two cleavage sites. The presence of a long prodomain in initiator caspases enables interaction with adaptor proteins. One subgroup of initiator caspases, procaspase-8 and 10, has a DED in the prodomain and interacts with DED-containing adaptor proteins such as FADD and TRADD to mediate apoptosis induced by death receptors. Another subgroup contains a caspase recruitment domain (CARD) and will establish contact with adaptors such as Apaf1 that have a CARD to mediate apoptosis induced by mitochondria-related mechanisms. Procaspase-9 and 2 and CED-3 fall into this latter subgroup. The recruitment of pro-caspase by adaptors results in high local concentrations of procaspases that are adequate to induce self-proteolysis and trans-catalysis among recruited zymogens. The activated initiator caspases will subsequently cleave effector caspases

such as procaspase-3, 6 and 7. Thus, a cascade of proteolysis is initiated that will target multiple cellular substrates and lead to the progression of apoptosis (Earnshaw et al., 1999; Thornberry et al., 1997; Salvesen and Dixit, 1999).

e. Repression of apoptosis

As apoptosis plays important and pivotal physiological roles, negative regulation of apoptosis exists at multiple levels of apoptotic pathways to keep apoptosis in check. Decoy receptors exist to neutralize death signals and block apoptosis from the beginning. For example, DcR3, a homolog of Fas death receptor, contains no intracellular and transmembrane domain. It may act as a soluble decoy receptor to neutralize Fas ligand and block the initiation of apoptosis. Two other decoys, DcR1 and DcR2, also block DR4 and DR5 induced apoptosis (Sheikh and Fornace, Jr., 2000b).

Anti-apoptotic members of Bcl-2 family are negative regulators that block the initiation of mitochondria-mediated apoptosis. The mechanisms underlying this protection are still under investigation. However, anti-apoptotic Bcl-2 proteins are responsible for maintaining normal mitochondria functions including ATP production and exchange, membrane integrity and controlled permeability and stability of membrane potential (Adams and Cory, 1998).

FLIPs are a family of FLICE inhibitory proteins containing DED regions. One member, $FLIP_L$ resembles FLICE (procaspase-8) except for an inactive C-terminal catalytic domain. Thus, $FLIP_L$ can be recruited to a DISC complex through DED-DED interactions with adapter proteins and block the activation of caspase-8 and subsequent induction of apoptosis. Both viral and mammalian members of the FLIP family have been identified, indicating that invading viruses may use this mechanism to block apoptosis of

host cell and thus ensure the propagation of viruses (Krueger et al., 2001; Tschopp et al., 1998).

Inhibition of apoptosis at the caspase level is regulated through members of the inhibitors of apoptosis (IAP) family, characterized by baculoviral IAP repeats (BIR). BIR regions contain 70 amino acids with conserved space between cysteine and histidine and may represent a novel zinc-binding fold. Mammalian members of the IAP family include X-IAP, c-IAP-1, c-IAP-2 and survivin. IAPs act as caspase inhibitors by binding directly to activated caspase heterodimers and blocking their catalytic activities. Different members of the IAP family show different inhibitory profiles. For example, X-IAP, c-IAP-1 and c-IAP-2 bind and inhibit caspases-3, 7, and 9 but show no preference for caspases-1, 6, 8 and 10. Thus, these IAPs are more potent in blocking mitochondria-mediated apoptosis. Interestingly, Smac/DIABLO protein released from mitochondria during apoptosis seems to be a negative regulator of IAPs. Smac is encoded by nuclear genes and translated in the cytosol as a precursor protein. Upon transport into the inner space of mitochondria, Smac undergoes maturation by cleavage of its N-terminal targeting signal exposing a new Ala-Val-Pro-Ile terminus. This new N-terminus resembles a motif on procaspase-9 that mediates association with BIR regions of IAPs. Thus, upon entering the cytosol, Smac is able to compete with caspase-9 in the interaction with IAPs, and as a result, displaces IAP from caspase-9 and blocks the inhibitory effects of IAPs on caspase (Deveraux et al., 1997; Goyal, 2001).

Once inside host cells, viruses also produce potent protease inhibitors to block the initiation and execution phases of apoptosis. A cytokine response modifier protein, known as Crm A, is produced by the cowpox virus and is a potent inhibitor of granzyme

B and caspases-8 and to a lesser extent caspases-10, 3 and 7. Caspase-9 is insensitive to Crm A. Another viral protease inhibitor from baculovirus, p35, shows broad-spectrum inhibition on all caspases with similar efficiency (Komiyama et al., 1996; Ekert et al., 1999; Bortner and Cidlowski, 2002).

f. Caspase substrates and the execution phase of apoptosis.

Although the initiation stage of apoptosis is incredibly complex, all signaling pathways appear to converge at the execution phase. As a result, cells undergoing apoptosis in response to various stimuli show common morphological and biochemical characteristics with only a few exceptions. The execution phase of apoptosis lasts only a short period of time. One model divides the execution stage of apoptosis into release, blebbing and condensation stages based on the progression of morphological changes (Mills et al., 1999).

Release of adherent cells from the substratum involves detachment from the extracellular matrix and reorganization of focal adhesions due to actin rearrangement. Proteolytic cleavage of components of focal adhesion processes, such as FAK (focal adhesion kinase), talin, α-actinin and p130-CAS is thought to mediate the detachment of dying cells. Pak2 (p21-activated kinase 2), a protein known to reorganize the actin cytoskeleton, is also cleaved by caspase to become constitutively active (Hacker, 2000; Rudel and Bokoch, 1997). Membrane blebbing is controlled by actin-myosin II contraction. A ROCK I (Rac-associated kinase) kinase shown to be a key regulator of membrane blebbing is also cleaved by caspase to become active during apoptosis (Coleman et al., 2001). Formation of apoptotic bodies and condensation of cellular contents represent the final execution stage of apoptosis. Pak1 protein plays critical roles

in the formation of apoptotic bodies (Mills et al., 1999). Tissue transglutaminase (tTG) is responsible for cytoplasmic compaction. During apoptosis, tTG expression increases and mediates the assembly of a highly stabilized and cross-linked intracellular protein net through the formation of ε(γ-glutamyl)lysine crosslinks between proteins. This limits the leakage of cellular contents and avoids the provocation of inflammation (Lesort et al., 2000; Chen and Mehta, 1999).

Morphological and chemical changes in the nucleus during apoptosis are mediated by caspase activities through cleavage of lamins and PARP and activation of endonucleases. CAD, known as caspase-activated DNase, is expressed along with ICAD, an inhibitor of CAD. Under normal conditions, CAD is inactive through ICAD binding. Caspases catalyze the cleavage of ICAD and release its inhibitory effect on CAD. CAD then functions in the DNA fragmentation process (Bratton, 1994). Two other proteins, EndoG and AIF, can mediate DNA fragmentation after their release from mitochondria. EndoG induces nucleosomal DNA fragmentation independent of activation of caspases. AIF, a flavoprotein, is able to induce chromatin condensation and large-scale DNA fragmentation when translocated to the nucleus. It remains unclear whether AIF activity also requires activation of caspases (Wang, 2001). Cleavage of lamins is required for disassembly of the nucleus into membrane-enclosed vesicles and PARP inactivation will block DNA repair to ensure initiation of apoptosis (Hacker, 2000).

g. Caspase-independent apoptosis

Although the majority of apoptosis is dependent on activation of caspases, new information is accumulating to support the existence of caspase-independent apoptosis. Cells from Apaf-1 knockout mice undergo apoptosis in response to serum withdrawal and

other stress signals in the absence of caspase activation (Honarpour et al., 2000). Both AIF and EndoG can induce apoptotic nuclear changes without the involvement of caspase activity (Lorenzo et al., 1999; Wang, 2001). Other non-caspase proteases, such as calpains, mediate some aspects of apoptosis (Kidd et al., 2000; Squier et al., 1999; Squier and Cohen, 1997). Calpains are a family of Ca^{2+}-stimulated cysteine proteases that are ubiquitously expressed (Carafoli and Molinari, 1998). Disturbance of calcium homeostasis during apoptosis may lead to the activation of Ca^{2+}-dependent calpains. There is evidence that calpain functions in a caspase-independent manner to promote apoptosis (Wood et al., 1998; Wolf et al., 1999; Villa et al., 1998). Calpains also regulate caspase-dependent apoptosis and play roles either upstream or downstream of caspase activation (Wood and Newcomb, 1999; Waterhouse et al., 1998).

4. Physiological significance

a. Homeostasis of multicellular organisms

Every day millions of cells in the human body die as a result of apoptosis. A balance of cell proliferation and cell death is essential for maintaining life and normal cell turnover depends on apoptosis. Circulating blood cells have a short life-span and senescent blood cells undergo apoptosis rather than necrosis to ensure rapid and safe removal (Zwaal and Schroit, 1997). Apoptosis is also crucial for the appropriate development of multicellular organisms. During the development of *C. elegans*, 131 of a total of 1090 cells are eliminated through programmed cell death as the organism reaches maturation (Meier et al., 2000). Knockout studies of key apoptotic regulators in animal models confirm the direct involvement of apoptosis in regulating development. Bax and Bak double knockout mice die prematurely with massive defects in embryonic

development (Lindsten et al., 2000). Apaf-1 knockout mice, as well as mice lacking expression of caspase-9 or caspase-3, showed different extents of defects in brain development (Yoshida et al., 1998; Kuida et al., 1998; Kuida et al., 1996). Several developmental functions have been suggested to be mediated by apoptosis, including sculpturing structure, deleting unwanted cells, eliminating abnormal or misplaced cells and adjusting cell numbers (Jacobson et al., 1997). Apoptosis also has important functions in the immune system as it regulates the maturation of B and T-lymphocytes and the elimination of infected cells by cytotoxic T lymphocytes (Krammer, 2000).

b. Apoptosis and human diseases

Due to the physiological importance of apoptosis, malfunctions in the regulation of the apoptotic machinery resulting in too much or too little cell death will lead to serious human diseases. Too little apoptosis appears to be the main cause of certain human autoimmune diseases, such as autoimmune lymphoproliferative syndrome (ALPS) and systemic lupus erythematosus (SLE). ALPS patients show profound defects in lymphocyte apoptosis as a result of direct mutation in Fas or downstream caspase-10. Patients display progressive lymphoproliferation associated with autoimmunity and accumulation of unwanted T-lymphocytes in the peripheral lymphoid system. Soluble Fas found in some SLE patients may function as a decoy to neutralize Fas and inhibit Fas-mediated apoptosis. Defects in removing debris from apoptotic cells are among the causes of SLE (Fadeel et al., 1999a).

Reduced rates of apoptosis can induce cancer and many key regulators of apoptosis were first identified as proto-oncogenes. Bcl-2 was originally identified as an oncogene enriched in B cell lymphomas and it is now known to be an inhibitor of apoptosis. p53 is

a tumor suppressor and mutations in this protein contribute to the formation of many human tumors and cancers; p53 is a key mediator in initiating stress- and DNA damage-mediated apoptosis. Anti- and pro-apoptotic members of the Bcl-2 family, as well as many death receptors, are p53-induced proteins with their expression controlled by p53. Some mutations in p53 lead to reduced apoptosis even when genome integrity is severely damaged. Both the p53 and the Bcl-2 families are prime targets for finding an effective cure to cancer (Fadeel et al., 1999a).

Too much or uncontrolled apoptosis may lead to neurodegenerative diseases. Massive apoptotic elimination of neuron cells occurs during the normal development of the nervous system but after reaching maturation, neuronal cells normally become relatively inactive in proliferation and apoptosis. Improper neuronal cell death is a feature of a variety of neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease, Parkinson's disease and frontotemporal dementia. AD is the most common form of adult-onset dementia. β-amyloid peptide produced in AD patients is a main factor in the disease and *in vitro* assays indicate that β-amyloid can induce apoptosis of neuronal cells (Nishimoto et al., 1997; Yuan and Yankner, 2000).

Knowledge of apoptosis may also help combat viral and bacterial infection. Pathogens use self-made negative regulators of apoptosis to block apoptosis in host cells while promoting apoptosis in defense cells recruited to infection sites as part of the immune response.

The occurrence of apoptosis can be associated with most human pathologies and in some cases, apoptosis is the main cause of disease. Accordingly, effective therapeutic strategies may be developed by modulating the process of apoptosis. In other cases,

cellular apoptosis is a natural response to stress signals from other pathogens. Thus, targeting apoptosis as an approach to clinical intervention may only reduce symptoms rather than providing a final cure. Nonetheless, a clearer understanding of apoptosis has broad-ranging implications for intervention in many serious diseases.

B. Metabolism of PtdSer in mammalian cells

As mentioned previously, one of the characteristics of apoptosis is an alteration of asymmetry distribution of aminophospholipids in the plasma membrane resulting in the appearance of PtdSer on the outer surface of cells and apoptotic bodies. To better appreciate the role of this component of the apoptotic process, it is important to understand how PtdSer biosynthesis and movement within the cell are regulated during apoptosis.

1. *De novo* biosynthesis of PtdSer

When comparing among bacteria, yeast and mammalian cells, distinctive differences in the biochemical reactions involved in *de novo* synthesis of PtdSer have been identified. In bacteria and yeast cells, PtdSer is synthesized through reactions between CDP-diacylglycerol and L-serine catalyzed by PtdSer synthase (Yamashita and Nikawa, 1997; Matsumoto, 1997); however, this biosynthetic pathway does not exist in mammalian cells (Bjerve, 1973). Early studies using rat liver extracts showed that radiolabeled L-serine is converted to phospholipids in the presence of calcium. A two-step reaction whereby serine first displaces the ethanolamine headgroup from phosphatidylethanolamine (PtdEtn) to form PtdSer with release of free ethanolamine was suggested (Fig. 3). PtdSer can then be decarboxylated to produce PtdEtn and CO_2. The net result of this process is the production of ethanolamine from serine with PtdSer as an intermediate

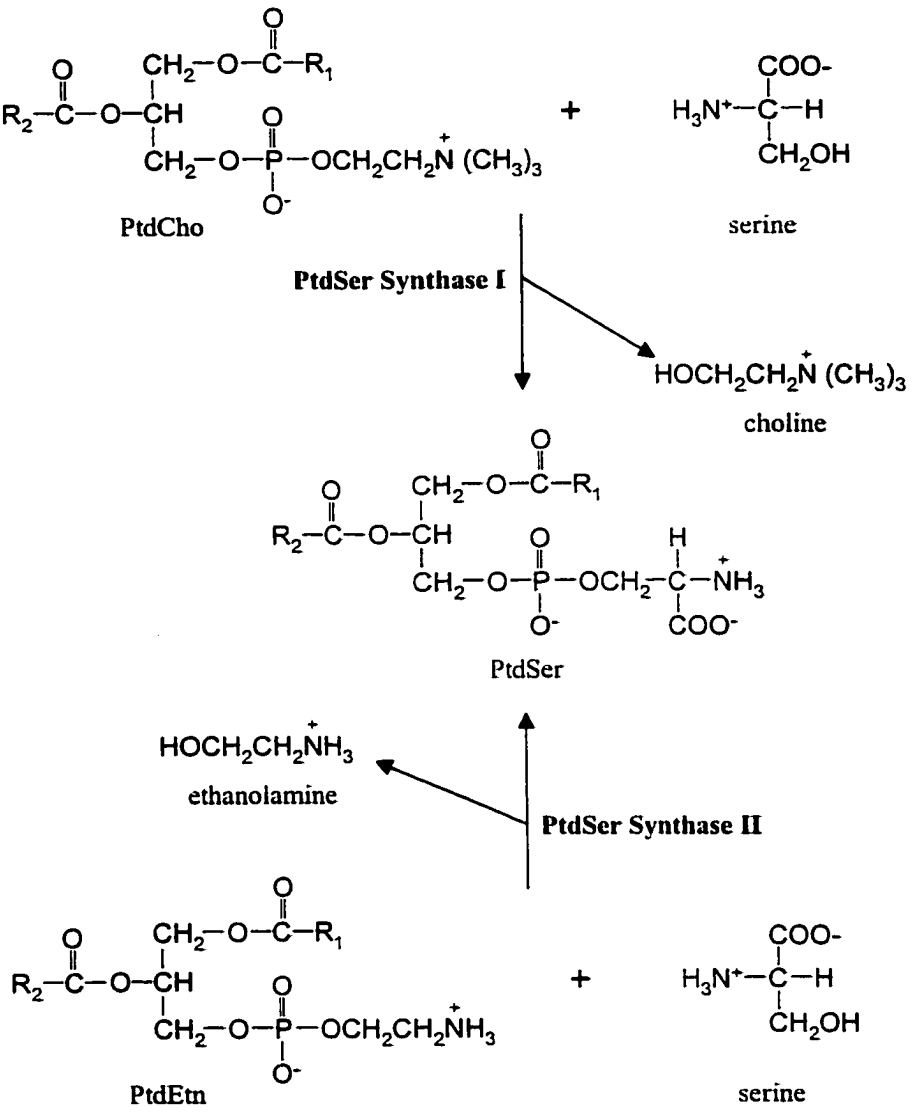

Figure 3. Biosynthesis of PtdSer in mammalian cells through serine base-exchange reactions. Phosphatidylserine (PtdSer) is synthesized by a Ca^{2+}-stimulated and energy-independent exchange of L-serine with the head groups of existing phospholipids. Serine base-exchange reactions are catalyzed by two isoforms of PtdSer synthase (PSS) *in vivo*; PSS I converts phosphatidylcholine (PtdCho) to PtdSer whereas PSS II utilizes phosphatidylethanolamine (PtdEtn) as substrate for PtdSer formation.

(Borkenhagen et al., 1961). Through further experiments, base-exchange reactions have been described between free L-serine, choline, ethanolamine and the base moiety of existing phospholipids. Whereas phosphatidylcholine (PtdCho) and PtdEtn are derived from CDP-choline or CDP-ethanolamine pathways, Ca^{2+}-dependent base-exchange reactions are the main source of PtdSer formation in mammalian cells (Hübscher, 1962). (Bell and Coleman, 1980).

The identification of mutants with defects in PtdSer formation have helped to clarify details of PtdSer biosynthesis pathways and have validated that PtdSer is crucial for the survival of mammalian cells. Several PtdSer auxotrophy mutants, including m64 (Kuge et al., 1985) and PSA-3 (Kuge et al., 1986a), have been isolated from mutagenized CHO-K1 cells. Similar defects have been reported for a mutant (M.9.1.1) isolated from CHO-K1 cells in another laboratory (Voelker and Frazier, 1986). In general, these PtdSer synthesis mutants only grow when the medium is supplied with PtdSer or ethanolamine. Cellular levels of PtdSer and PtdEtn are decreased by 50% and 70%, respectively, in these mutants when they are cultured in medium without supplementation, due to a failure to utilize PtdCho as precursor for PtdSer biosynthesis.

A putative ethanolamine base-exchange enzyme purified from rat liver is a 100 kDa protein with high ethanolamine and serine base-exchange activities (Suzuki and Kanfer, 1985). *In vitro* assays show that this protein converts PtdEtn to PtdSer efficiently whereas PtdCho and other phospholipids cannot be used as acceptors for serine residues. Further mutagenesis with PSA-3 cells has resulted in a double mutant PSB-2 in which levels of PtdSer formation are only 5% those detected in wild type CHO-K1 cells (Saito et al., 1998). The growth defect in PSB-2 can be restored by supplementation with PtdSer but

not with ethanolamine. The defect identified in PSB-2 cells is an inability to convert PtdEtn to PtdSer in addition to an inability to use PtdCho as a substrate for PtdSer synthesis. Thus, it was proposed that PtdSer biosynthesis in mammalian cells is catalyzed by two base-exchange enzymes: a PtdSer synthase I (PSS I) that uses PtdCho or PtdEtn to form PtdSer *in vitro* (but only converts PtdCho to PtdSer in intact cells) and PtdSer synthase II (PSS II) that only converts PtdEtn to PtdSer, both *in vitro* and *in vivo* (Fig. 3).

PtdSer biosynthesis involves a series of sequential reactions, starting with a PSS I-catalyzed base-exchange reaction between PtdCho and L-serine to form PtdSer (reaction 1). PtdSer can be directed to incorporation into cellular membranes (such as plasma membrane) or can be rapidly decarboxylated to give rise to PtdEtn through the activity of PtdSer decarboxylase (reaction 2). PtdEtn may then be converted back to PtdSer through a base-exchange reaction catalyzed by PSS II (reaction 3).

PtdCho + L-serine →PtdSer + choline (1)

PtdSer → PtdEtn + CO_2 (2)

PtdEtn + L-serine → PtdSer + ethanolamine (3)

The net result of these three reactions is the generation of PtdSer, ethanolamine and choline from PtdCho and two L-serine molecules. Ethanolamine is a by-product of PtdSer biosynthesis and this explains why ethanolamine supplementation is not needed in most mammalian cell cultures. PSS I (reaction 1) is defective in PSA-3, mutant 64 and M.9.1.1 cells and both PSS I and II (reactions 1 and 3) are expressed at low levels in PSB-2 cells.

Initial subcellular fractionation studies indicate that PtdSer biosynthesis takes place mainly in the endoplasmic reticulum (ER) isolated as microsomes (Fig. 4) (Dennis and

Kennedy, 1972; Bjerve, 1985; Bjerve, 1973). Overall activity is predominantly distributed through the rough ER as the specific activity for overall synthesis of PtdSer from serine is 4-fold higher there than in the smooth ER. Base-exchange activities are also found in nuclei (Jelsema and Morre, 1978). This has led to speculation that base-exchange reactions might occur on ribosomes but there is no direct evidence supporting this (van Golde et al., 1974). It is now generally accepted that base-exchange activities are enriched in the ER and in mitochondria-associated membranes (MAM). MAM, initially known as fraction X, is a membrane fraction co-isolated along with the bulk of mitochondria after centrifugation. MAM can be separated from mitochondria on Percoll gradients (Vance, 1990). This transitional compartment of membranes, proposed to function between mitochondria and other membrane regions of the cell such as ER, nuclei and plasma membrane, has higher base-exchange specific activity than that of ER.

2. Characteristics of enzymes catalyzing PtdSer formation

a. Cloning of *pss A* gene and identification of PSS I

PSS I, known as base-exchange enzyme I, is defined as an enzyme that has serine, choline and ethanolamine base-exchange activities *in vitro* and utilizes PtdCho to form PtdSer in intact cells (Kuge et al., 1986b). PSA-3 cells are completely devoid of PSS I activity. Genetic complementation of PSA-3 cells with a cDNA library from wild-type CHO-K1 cells facilitated the isolation of a 1.5 kDa cDNA fragment encoding a 471 amino acids transmembrane protein essential for PSS I activity (Kuge et al., 1991).

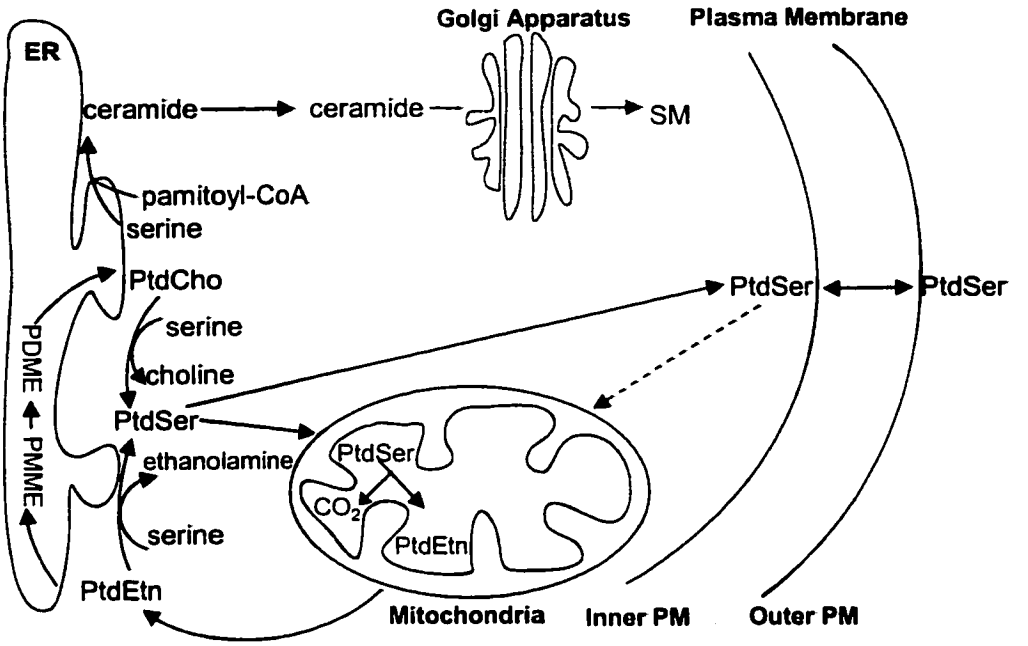

Figure 4. Synthesis of serine-derived phospholipids and interorganelle transport of PtdSer. Conversion of PtdCho into PtdSer catalyzed by PSS I occurs on endoplasmic reticulum (ER) or mitochondria-associated membranes (MAM). PtdSer is delivered to the outer mitochondrial membrane through contacts between MAM and mitochondria. PtdEtn is formed by decarboxylation of PtdSer in the inner mitochondrial membrane and is rapidly transported back to ER and MAM to be converted to PtdSer or to be methylated to PtdCho (with PMME (monomethyl-PtdEtn) and PDME (dimethyl-PtdEtn) as intermediates). These reactions result in the formation of PtdSer and ethanolamine at the expense of PtdCho and serine. Newly synthesized PtdSer is transported to the plasma membrane through vesicular transport or other unidentified mechanisms. Serine can also be used in the formation of ceramide on the ER which is then delivered to the Golgi apparatus for sphingomyelin (SM) synthesis.

Transfection of PSA-3 cells with this cDNA enables the conversion of PtdCho to PtdSer and restores cell growth and levels of PtdSer and PtdEtn in the absence of PtdSer or ethanolamine supplementation. The cDNA originating from CHO cells is termed *pss A*.

Two polyclonal antibodies were generated against the N- and C-terminal regions of *pss A* protein based on the predicted amino acid sequences (Saito et al., 1996). Immunoblotting with these antibodies indicates the existence of a protein with apparent molecular weight of 42 kDa in CHO-K1 cells and CDT-1 cells generated by over-expressing *pss A* in PSA-3 cells. Antibodies raised against *pss A* protein inhibited serine base-exchange activity of cell extracts from CDT-1 cells in a concentration-dependent manner. Thus, the *pss A* gene product is identified as the PtdSer synthase (PSS I) of CHO-K1 cells. Based on the *pss A* gene originally isolated from hamster cells, cDNA encoding PSS I has been identified in mouse and human cells (Stone et al., 1998; Nomura et al., 1994). PSS I proteins from different species share very high homology with more than 90% identical amino acids.

b. Cloning of *pss B* gene and identification of PSS II

Based on the assumption that PSSI and PSS II might share sequence homology, a human EST was isolated and found to have 30% homology with the *pss A* gene using a GenBank search. A hamster counterpart has been cloned subsequently by polymerase chain reaction using primers designed from the human EST. This cDNA fragment, termed *pss B*, encodes a protein of 474 amino acids and shares 32% identity in amino acid sequence with PSS I protein (Fig. 5) (Kuge et al., 1997). Transient expression of the *pss B* gene in CHO-K1 cells resulted in a significant increase in both serine and

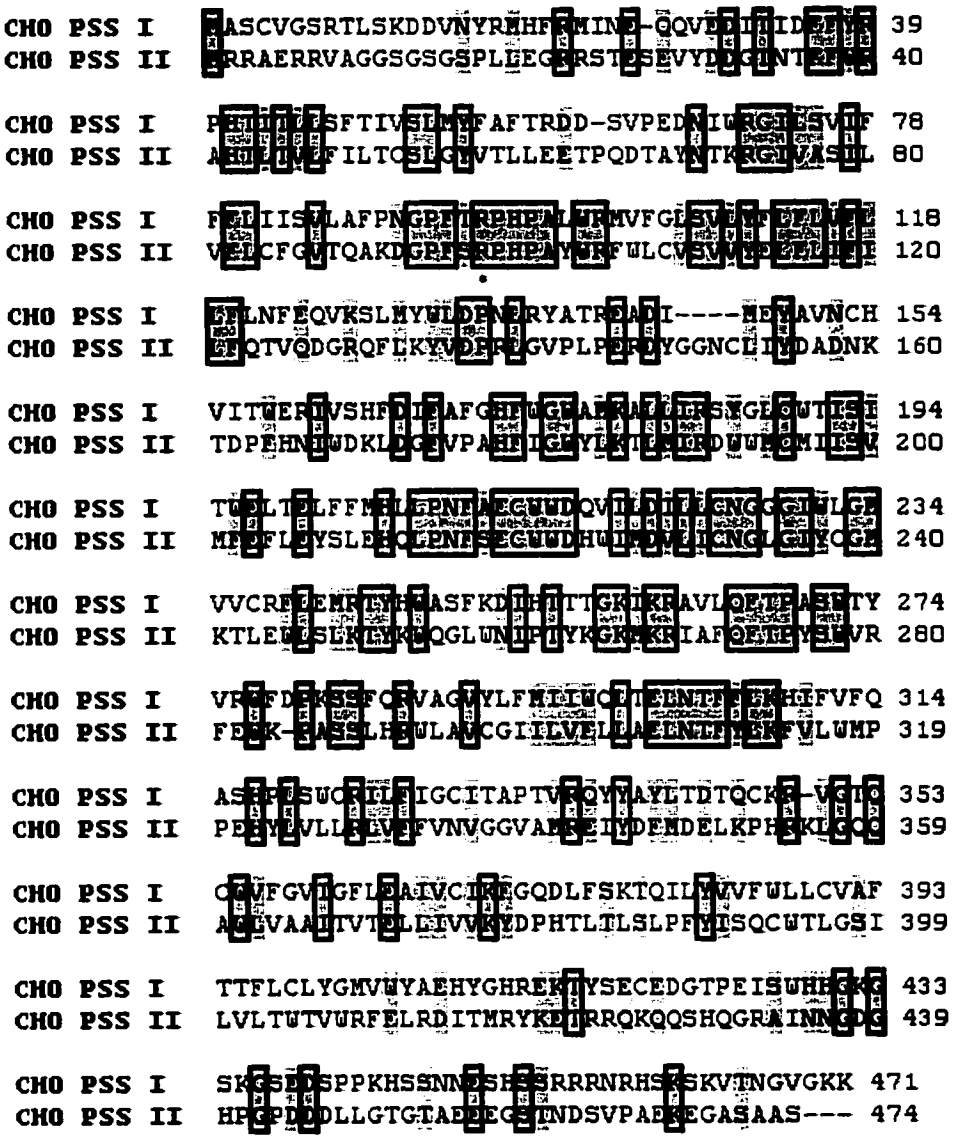

Figure 5. Comparison of protein sequences of phosphatidylserine synthase isoforms from CHO-K1 cells. Predicted protein sequences of PSS I (GenBank accession number A41680) and PSS II (GenBank accession number BBA20355) from CHO-K1 cells were aligned using CLUSTALW. Identical amino acids are shown in outlined boxes, and conservative substitution are on a gray background.

ethanolamine base-exchange activities whereas no change in choline base-exchange activity was observed. This is consistent with the fact that PSS II can only catalyze serine and ethanolamine base-exchange reactions *in vitro* (Kuge et al., 1997). To further confirm the *pss B* product as PSS II, a double-mutant PSB-2 cell line was derived from PSA-3 cells lacking PSS I (Saito et al., 1998). Northern blot analysis with a *pss B* specific probe indicated only 10% expression of *pss B* gene in PSB-2 cells compared to PSA-3 cells and endogenous PtdSer content was only 5% that of CHO-K1 cells. Unlike PSA-3 cells that can grow normally in medium supplemented with PtdSer, PtdEtn or free ethanolamine, the growth of PSB-2 can only be restored by the addition of PtdSer to the medium. This indicates that PtdSer cannot be formed from PtdEtn in PSB-2 cell. When the *pss B* gene is stably expressed in PSB-2 cell, the formation of PtdSer through PtdEtn is restored whereas the conversion from PtdCho to PtdSer is still missing. Similarly PSB-2 cells expressing *pss B* product can regain a normal growth rate in PtdEtn supplemented medium. Thus, the *pss B* gene encodes the PSS II protein which is responsible for catalyzing the serine base-exchange activity that converts PtdEtn to PtdSer in intact cells (Saito et al., 1998). By using primers designed from the hamster *pss B* gene, a murine PSS II cDNA has also been isolated as a 1.4 kb fragment encoding a 473 amino acids PSS II (Stone and Vance, 1999).

c. Properties of PSS I and PSS II

PSS I is a highly hydrophobic protein with five predicted transmembrane domains. The discrepancy observed between the theoretical (55.3 kDa) and apparent (42 kDa) molecular weights of PSS I may be explained by an increase in SDS binding during SDS-PAGE as a result of the high content of hydrophobic amino acids (Saito et al., 1996). No

obvious functional domains have been identified in the PSS I sequence except for a highly conserved motif containing an arginine residue at position 95 and surrounding amino acids. It is postulated that Arg 95 plays crucial role in regulating PSS I activity in response to PtdSer levels (Kuge et al., 1998). Immunochemical studies show that PSS I is enriched in microsomes and MAM (Saito et al., 1996). Physically, MAM membranes are closely related to ER membranes and they share several protein markers including glucose-6-phosphatase. This marker is 2-fold enriched in MAM compared to ER (Vance, 1990). There are multiple reports that the specific activity of the serine base-exchange reaction is more enriched in MAM (2-fold) than in ER (Shiao et al., 1995; Saito et al., 1996; Stone and Vance, 2000). Choline base-exchange activity unique to PSS I appears to be present exclusively in MAM preparations and immunoblotting confirms that the 42 kDa PSS I protein is located primarily in MAM (Stone and Vance, 2000). Two lysine residues (-Lys-Lys-COOH) are found in the C-terminus of PSS I protein. Accordingly, this motif shares similarity with ER-targeting sequences such as - Lys-X-Lys-XX-COOH and -XX-Lys-Lys-XX-COOH (Jackson et al., 1990). The di-lysine motif has been proposed as an ER retention signal for PSS I; however, deletion of the di-lysine residues at C-terminus does not alter the MAM localization of PSS I indicating that these amino acids do not serve as a MAM targeting signal (Stone and Vance, 2000).

PSS II protein also is highly hydrophobic having a similar distribution of transmembrane domains compared to PSS I. In contrast to PSS I, this protein migrates to a 52 kDa position, close to its calculated molecular weight (55.3 kDa) (Stone and Vance, 2000), implying different interactions with SDS. Comparison of the two proteins indicates a conserved motif surrounding Arg95 of PSS I and Arg 97 of PSS II (Fig. 5).

This motif is involved in the regulation of PtdSer formation by both proteins. PSS II protein does not have a C-terminal di-lysine motif but it contains a double arginine motif, NH_2-Met-Arg-Arg-Alu-Glu. This also is known to be an ER targeting motif (Schutze et al., 1994). PSS II also appears to be exclusively localized to MAM similar to PSS I (Stone and Vance, 2000). As both ethanolamine and serine base-exchange activities also have been detected on the ER, the possibility is raised of the existence of another ER-specific PtdSer synthase.

d. Regulation of PSS I and PSS II activities

As little predicted information has so far been deduced from the protein structures of PSS I and PSS II, knowledge about regulation of PtdSer biosynthesis through these two enzymes is quite limited. Similar to most biochemical reactions, PtdSer formation is likely to be regulated by substrate availability, product concentrations, enzyme expression, degradation and modification, availability of co-factors, and spatial co-localization of components involved.

(1) PtdSer-mediated feedback inhibition

PtdSer-mediated inhibition of PtdSer biosynthesis is the main regulatory mechanism known so far. When grown in medium containing PtdSer, CHO-K1 cells incorporate and distribute exogenous PtdSer quickly inside the cells. As a result, serine incorporation into PtdSer is completely blocked (98%), whereas the PtdSer mass remains the same (Nishijima et al., 1986). Furthermore, when PSS I is over-expressed in CHO-K1 cells, serine incorporation into PtdSer and levels of PtdSer show little difference from parent cells in spite of increased levels of PSS I proteins (Kuge et al., 1998). PtdSer is indispensable for cell growth and cells tend to maintain a constant level of PtdSer by

feedback inhibition mediated by PtdSer. PtdSer directly inhibits PSS I activity *in vitro* in a concentration-dependent manner. Arg-95 of PSS I is crucial in mediating this inhibition as a mutation of Arg to Lys (R95K) results in total elimination of PtdSer inhibition. Transient expression of an R95K mutant in CHO-K1 cells resulted in a 5-fold higher PtdSer biosynthetic rate relative to control cells in the absence of PtdSer. This indicates that PtdSer biosynthesis is inhibited by feedback control even under basal conditions. Addition of PtdSer to the growth medium will not block PtdSer biosynthesis in R95K mutant cells as seen in control cells. Thus, it seems likely that PtdSer directly regulates PSS I activity through Arg-95 in CHO-K1 cells.

PtdSer biosynthesis through PSS II activity also is inhibited by PtdSer. Conversion of PtdEtn to PtdSer catalyzed by PSS II is reduced by ~95% upon addition of PtdSer to CHO-K1 cells. However different mechanisms may be involved in PtdSer-mediated inhibition of PSS II compared to that of the PSS I enzyme. First, in CHO-K1 cells over-expressing PSS II, addition of exogenous PtdSer only reduces PtdSer biosynthesis by 35% whereas nearly complete (98%) inhibition is observed in both parent and PSS I expressing CHO-K1 cells. Secondly, although PSS I over-expression does not change PtdSer levels relative to parent cells, a 1.6-fold increase in PtdSer composition occurs in PSS II expressing cells. Thirdly, in contrast to direct inhibition of PSS I by PtdSer *in vitro*, PSS II protein is insensitive to PtdSer at various concentrations *in vitro*. One feasible explanation is that PtdSer inhibits PSS II activity through an intermediate molecule. PtdSer may bind to the putative co-factor and then inhibit PSS II activity by direct interaction between these two proteins. This could explain why PSS II itself does not respond to PtdSer and why cells over-expressing PSS II seem to be leaky in PtdSer-

mediated inhibition. Inhibition by PtdSer may be limited by the availability of the putative co-factor. PSS II proteins may be produced in excess, while expression of the co-factor remains unchanged. Once all co-factors are saturated by over-expressed PSS II, PtdSer-mediated inhibition of PSS II is lost (Kuge et al., 1999).

Regulation of PtdSer biosynthesis by PtdSer is complex. Different cellular conditions may result in different regulatory mechanisms for PSS I and PSS II proteins. Over-expression of hamster PSS I in CHO-K1 cells does not increase the PtdSer biosynthetic rate and levels of PtdSer accordingly, indicating a tight feedback inhibition on PSS I activity (Kuge et al., 1998). However, when murine PSS I is over-expressed in McArdle 7777 rat hepatoma cells or M.9.1.1 cells (a PSS I defective mutant derived from CHO cells), the biosynthetic rate of PtdSer increases 3-fold implying that PSS I is not regulated by end-product inhibition in these cases. These cells maintain a constant level of PtdSer by increased PtdSer catabolism through decarboxylation. Concurrent inhibition of the CDP-ethanolamine pathway results in unchanged levels of PtdEtn also (Stone et al., 1998). PtdSer biosynthesis through murine PSS II is regulated by end-product inhibition since over-expression of murine PSS II in McArdle and M.9.1.1 cells does not enhance the PtdSer biosynthetic rate and overall levels of PtdSer in intact cells (Stone and Vance, 1999). Furthermore, increased PSS II activity does not result in inhibition of the CDP-ethanolamine pathway.

PSS I and PSS II show differential tissue expression which may indicate unique functions for both enzymes. Northern blot analysis indicates that PSS I is widely expressed in most tissues whereas PSS II is abundant only in testis (Stone and Vance, 1999). Using a more sensitive RT-PCR technique, PSS I mRNA is detectable in all

tissues examined and is enriched in kidney, liver, brain, heart, lung and testis. PSS II mRNA is expressed most abundantly in testis and at high levels in kidney and brain but is not detectable in spleen and adipose tissue (Sturbois-Balcerzak et al., 2001).

(2) Calcium-dependent PtdSer biosynthesis

Serine base-exchange reactions occur independent of energy, but require high (in the millimolar range) concentrations of Ca^{2+} in the ER lumen (Rakowska and Wojtczak, 1995). Fluctuations in Ca^{2+} levels in the cell, especially in ER stores, influence the biosynthesis of PtdSer (Pelassy et al., 1992a). Serine base-exchange enzymes (PSS I or PSS II), may contain an active site for Ca^{2+} at the luminal surface of the ER membrane whereas serine is accessible for the transmembrane enzymes from the cytoplasmic surface of the ER (Wiktorek et al., 1996). Thus, depletion of intracellular Ca^{2+} stores in intact cells results in the diminution of PtdSer biosynthesis. Agents known to alter the intracellular levels of Ca^{2+}, such as Ca^{2+} ionophores or chelators, inhibitors of the endoplasmic Ca^{2+}-ATPase (thapsigargin) and neurotransmitters (glutamate and acetylcholine), are effective inhibitors of PtdSer biosynthesis (Czarny et al., 1992; Pelassy et al., 1992a; Rakowska and Wojtczak, 1995).

PtdSer biosynthesis is regulated upon activation of cell surface receptors as an increase in cytosolic Ca^{2+} is triggered through signal transduction involving the production of inositol-1,4,5 triphosphate (IP_3) (Czarny et al., 1992; Mikhaevitch et al., 1994; Siddiqui and Exton, 1992). Receptor-mediated regulation of PtdSer synthesis is best characterized during T-cell activation. A strong inhibition of PtdSer synthesis is observed during T-cell activation induced with antibodies directed against T cell receptor complexes (TCR) (Didier et al., 1988). Protein tyrosine kinase, $p56^{lck}$, appears to be

activated by TCR complexes and to be responsible for regulating PtdSer biosynthesis. One mechanism might involve activation of phospholipase $C_{\gamma 1}$ ($PLC_{\gamma 1}$) through tyrosine phosphorylation mediated by $p56^{lck}$ (Marhaba et al., 1997). Activated $PLC_{\gamma 1}$ hydrolyses phosphatidylinositol bisphosphate (PIP_2) into two secondary messengers, diacylglycerol and IP_3, with the latter able to trigger the release of Ca^{2+} from ER stores. Depletion of intracellular Ca^{2+} stores inhibits *de novo* biosynthesis of PtdSer through unidentified mechanisms. Calmodulin (CaM), could be responsible for mediating the inhibition of PtdSer biosynthesis through a Ca^{2+}-responding protein kinase (Aussel et al., 1995a). It also has been suggested that CaM may interact directly with the serine base-exchange enzyme system provided that Ca^{2+} is present. CaM antagonists stimulate PtdSer biosynthesis and can reverse the inhibitory effects on PtdSer formation induced by T-cell activation (Aussel et al., 1995a; Pelassy et al., 2000a).

(3) Other regulation of PtdSer biosynthesis

Several agents have been implicated in the inhibition of PtdSer biosynthesis through Ca^{2+}-independent mechanisms. Caffeine inhibits PtdSer formation in many types of cells through direct effects on PtdSer synthases or by modification on PtdSer structures on the membrane (Pelassy et al., 1999). Hydrogen peroxide modulates the redox state of cells and greatly decreases serine incorporation into PtdSer; conversely, antioxidants, such as ascorbate or vitamin E, effectively stimulate PtdSer synthesis (Pelassy et al., 2001). An inhibitor of tyrosine phosphatase, orthovanadate, is also able to block PtdSer formation by bypassing T-cell activation pathways (Pelassy et al., 2000b). Thus, multiple mechanisms exist to regulate PtdSer biosynthesis *in vivo*. Elucidation of these complex processes will permit wider options for the modulation of PtdSer biosynthesis.

Cationic amphiphilic drugs (CADs), such as oleoylamine and stearoylamine, are molecules containing a hydrophobic acyl chain and a positively charged amino group. CADs specifically stimulate *de novo* PtdSer biosynthesis in synergy with Ca^{2+} ions. CAD-mediated increases in PtdSer formation are independent of the amount of Ca^{2+} present and can not be reversed by thapsigargin and EGTA (Aussel et al., 1995b). It is hypothesized that CADs may stimulate PtdSer biosynthesis by exposing the ethanolamine moiety of PtdEtn to a hydrophilic environment accessible to the catalytic site of the serine base-exchange enzymes (Singh et al., 1992a). Other amphiphilic compounds, including sphingosine, also play positive roles in regulating PtdSer biosynthesis and implicate the possible roles of positive charge in increasing PtdSer formation, (Singh et al., 1992b; Wiktorek-Wojcik et al., 1997).

K^+-channel blockers, such as quinine, also stimulate PtdSer (Aussel et al., 1990; Pelassy et al., 1992b). The stimulation is less specific to PtdSer and may be related to the modulation of lipid metabolism by these compounds.

In conclusion, cellular levels of PtdSer in mammalian cells appear to be under tight control. Cells try to maintain a constant level of PtdSer through differential regulation of PSS I or PSS II activities in a manner that is coordinated with the synthesis and turnover of other phospholipids to achieve a lipid homeostasis essential for cell survival. PtdSer-mediated inhibition of PtdSer biosynthesis is the primary known mechanism regulating PtdSer biosynthesis; other, yet unidentified, ways of regulation may exist.

e. PtdSer biosynthesis in nuclei and plasma membrane

In mammalian cells, MAM and ER membranes are the main sites of PtdSer formation; however, early studies indicated that serine base-exchange reactions also exist

in the nucleus and plasma membrane. In fact, the nuclear fraction has the highest specific activity even though the greatest total PtdSer synthetic activity is in microsomes (Jelsema and Morre, 1978). Studies using highly purified nuclei from rat liver have confirmed that nuclei have considerable capacity for Ca^{2+}-dependent PtdSer biosynthesis. Furthermore, nucleoplasts (from inner nuclear membranes) are the sites of PtdSer formation whereas serine base-exchange activity is absent in preparations of the outer membrane (Dygas et al., 2000). The identity of this nuclear "PtdSer synthase" is unclear though it may have an active site at the periplasmic side of the inner nuclear membrane. Nuclear biosynthesis of PtdSer may be physiologically relevant to the regulation of nuclear protein kinase C (PKC).

PtdSer is enriched in the plasma membrane of C6 glioma cells. PtdSer is 15% of the total phospholipids of plasma membrane compared to 6.5% in microsomes (Xu et al., 1994). After addition of radiolabeled serine to intact C6 glioma cells, newly synthesized PtdSer accumulates up to nearly half of its maximal levels in the plasma membrane by 30 min. The rapid accumulation of PtdSer on the cell surface, which may not be explained solely by transport from ER, supports the possibility of synthesis of PtdSer directly within the plasma membrane (Xu et al., 1994).

Local synthesis of PtdSer at the plasma membrane has been reported in plant cells (Vincent et al., 1999). Plant cells have two PtdSer synthetic pathways: a serine base-exchange reaction similar to that in mammalian cells and another reaction catalyzed by a CMP-phosphatidic acid (PA) 3'-phosphatidyltransferase that converts CMP-PA and L-serine into PtdSer and CMP. A high serine base-exchange activity, but not a

phosphatidyltransferase activity, is present in the plasma membrane and is responsible for the formation of PtdSer containing long or very long-chain fatty acids.

3. Interorganelle transport and catabolism of PtdSer

Regulation of PtdSer generation for membrane synthesis is complicated by the fact that PtdSer also is a metabolic intermediate. PtdSer is the end-product of the serine base-exchange reaction but also is a substrate for PtdEtn biosynthesis. In CHO-K1 cells and BHK-2 cells, PtdEtn is mainly formed through decarboxylation of PtdSer whereas the contribution from the CDP-ethanolamine pathway is negligible (Shiao et al., 1995; Voelker, 1984). Essentially all PtdEtn in mitochondria is formed from PtdSer. PtdEtn can be further methylated to form PtdCho (Vance et al., 1996). Special pools of serine-derived PtdSer, PtdEtn and PtdCho with unique functions appear to exist inside cells. In some cases, phospholipids derived from PtdSer are destined to secretory process and are preferentially assembled into lipoprotein (Vance and Vance, 1986; Vance, 1988; Vance, 1989).

a. Interorganelle transport of PtdSer

PtdSer biosynthesis is mainly catalyzed by PSS I and PSS II and occurs exclusively in MAM. Newly formed PtdSer is rapidly transported from MAM to the plasma membrane through unknown mechanisms. PtdSer decarboxylase is found only on the outer surface of the inner membrane of mitochondria. As a result, PtdSer has to be delivered from MAM to the surface of the outer mitochondria membrane and must migrate to the inner side of the outer membrane before delivery to the outer surface of the inner mitochondria membrane for decarboxylation. As PtdSer decarboxylase is not a rate-limiting enzyme, the formation of PtdEtn seems to be mainly regulated by the

interorganelle movement of PtdSer (Fig. 4). MAM is found in close proximity to the outer membrane of mitochondria and it is speculated that MAM comes into contact with the mitochondrial outer membrane. During this contact, PtdSer formed on MAM is assimilated into the acceptor membrane. A membrane protein on mitochondria is postulated to be involved in translocating PtdSer from MAM to mitochondria, although its identity remains a mystery (Shiao et al., 1998). Little is known about how PtdSer migrates to the inner mitochondrial membrane except that the process does not require ATP and is blocked by adriamycin and dinitrophenol (Voelker, 2000; Voelker, 1991). S100B protein, a 9 kDa protein from a family of small Ca^{2+}-binding proteins of the EF-hand type, has been identified with the ability to stimulate transport-dependent decarboxylation of PtdSer. As S100B does not stimulate PtdSer decarboxylase *per se*, it is thought to be a regulator of the transport of PtdSer to the mitochondrial inner membrane (Kuge et al., 2001). After formation on the inner mitochondrial membrane, serine-derived PtdEtn is rapidly distributed to other organelles, including ER and MAM, for membrane biogenesis. This redistribution process is poorly understood.

Intracellular trafficking of PtdSer to the plasma membrane is little understood but cytoplasmic proteins that bind and transfer PtdSer exist (Baranska and Grabarek, 1979). It is unclear whether PtdSer can be transported to the cell surface through protein-mediated pathways. In plant cells, PtdSer synthesized on the ER is delivered to the plasma membrane through a vesicular ER-Golgi-PM membrane pathway. In plant cells, PtdSer-rich vesicles (70-nm in size) are derived from ER through both biosynthetic pathways of PtdSer. Mammalian cells may utilize a similar vesicle-mediated mechanism to deliver PtdSer to the plasma membrane. Analysis of PtdSer composition of rat liver

subcellular membranes indicates that although PtdSer is synthesized in the ER (2.9%), it is more enriched in the transition vesicles (7.2%) operating between ER and the Golgi apparatus and most enriched in the plasma membrane (10.6%) (Moreau and Cassagne, 1994). Although there is no clear experimental evidence, PtdSer transport to the plasma membrane may be mediated through vesicular carriers (van Meer, 1989). However, direct contacts between ER and the plasma membrane may mediate the incorporation of ER-derived PtdSer into the acceptor membranes at the cell surface (Tilney et al., 2001).

b. PtdSer decarboxylation

Although PtdEtn can be formed in mammalian cells through two main pathways (the CDP-ethanolamine pathway and PtdSer decarboxylation), there is strong evidence indicating that PtdSer decarboxylation is responsible for the synthesis of the entire pool of PtdEtn in CHO cells. Only one PtdSer decarboxylase (PSD) has so far been cloned and characterized in mammalian cells. PSD is localized on the external side of the inner mitochondria membrane and its catalytic activity requires a pyruvoyl prosthetic group which is acquired post-translationally. PSD is translated as a 46 kDa precursor containing a mitochondrial transmembrane domain (TM) and an inner membrane sorting sequence (IMS). The large catalytic β-subunit is linked to the C-terminal small α-subunit through Leu-Gly-Ser-Thr. The proenzyme is sequentially processed to remove TM and IMS regions. The pyruvoyl prosthetic group is generated by a self-catalyzed endoproteolysis between the glycine and serine residues in the linker residues between α-and β-subunits which results in the formation of an amino terminal pyruvate on the α subunits (Voelker, 1997).

C. PtdSer externalization during apoptosis

1. Lipid asymmetry on plasma membrane and its maintenance

Plasma membranes of eukaryotic cells are composed of two layers of lipids with phospholipids and cholesterol making as much as 72% of the total membrane lipid (Cullis et al., 1996). PtdSer constitutes 5-10% of total membrane lipids and is a relatively minor component of the plasma membrane (Voelker, 1996); however, it plays essential roles in cell growth and survival (Kuge et al., 1986a). A key characteristic of bio-membranes is the asymmetric distribution of lipid components. Aminophospholipids such as PtdSer and PtdEtn are mostly confined to the inner leaflet, whereas choline-containing phospholipids such as PtdCho and sphingomyelin (SM) are enriched in the outer layer (Bretscher, 1972). Asymmetric distribution of lipids provides cells with two physiochemically distinct membrane layers. The outer surface is packed tightly due to interactions between choline-containing phospholipids and cholesterol (Seigneuret et al., 1984; Morrot et al., 1986); the inner layer has greater fluidity for membrane invagination involved in the formation of endocytotic vesicles due to the greater presence of aminophospholipids (Farge et al., 1999).

PtdSer is an anchor point for cytoskeletal proteins such as fodrin (Williamson et al., 1987; Haest et al., 1978) and is a regulator for signaling molecules such as PKC (Nishizuka, 1992) and myristoylated alanine-rich C kinase substrate (MARCKS) (Taniguchi and Manenti, 1993). Thus, PtdSer plays important roles in regulating both the structural integrity of the cell and intracellular signal transduction. On the plasma membrane, spontanous membrane lipid migration occurs at a slow rate. To establish and maintain lipid asymmetry, energy is required and specific regulatory proteins are

involved. Three kinds of lipid transporters have been described (Fig. 6): aminophospholipid translocases, phospholipid scramblases and floppases (Diaz and Schroit, 1996; Bevers et al., 1999).

a. Aminophospholipid translocase (flippase)

Aminophospholipid translocase (APT), first reported in 1984 (Seigneuret and Devaux, 1984), is an ATP-dependent lipid transporter with Mg^{2+}-ATPase activity that moves lipids from the outer leaflet to the inner layer (Bevers et al., 1999). ATP hydrolysis occurs during the transport cycle and one molecule of ATP is consumed for each aminophospholipid transported (Daleke and Lyles, 2000). APT is inhibited by Ca^{2+} and vanadate (an inhibitor of many phosphatases and transport ATPases) and also is sensitive to histamine reactive compounds or reagents that oxidize or alkylate (N-ethylmaleimide) protein sulfhydryls (Moriyama and Nelson, 1988; Connor et al., 1992). When cysteines in APT are oxidized by reactive oxygen species, the activity of APT is completely inhibited (Herrmann and Devaux, 1990; de Jong et al., 1997). APT has specificity for aminophospholipids, with higher preference for PtdSer than for PtdEtn with the substrate specificity of APT determined by both the headgroup and the diacylglycerol back bone of the phospholipid. Thus, glycero-phosphoserine, but not L-serine or phosphoserine, competitively inhibits PtdSer transport by APT (Daleke and Lyles, 2000). This specificity for aminophospholipid movement plays a major role in maintaining lipid asymmetry. Under normal conditions when ATP is constantly generated and cellular calcium levels are low, inward movement catalyzed by APT

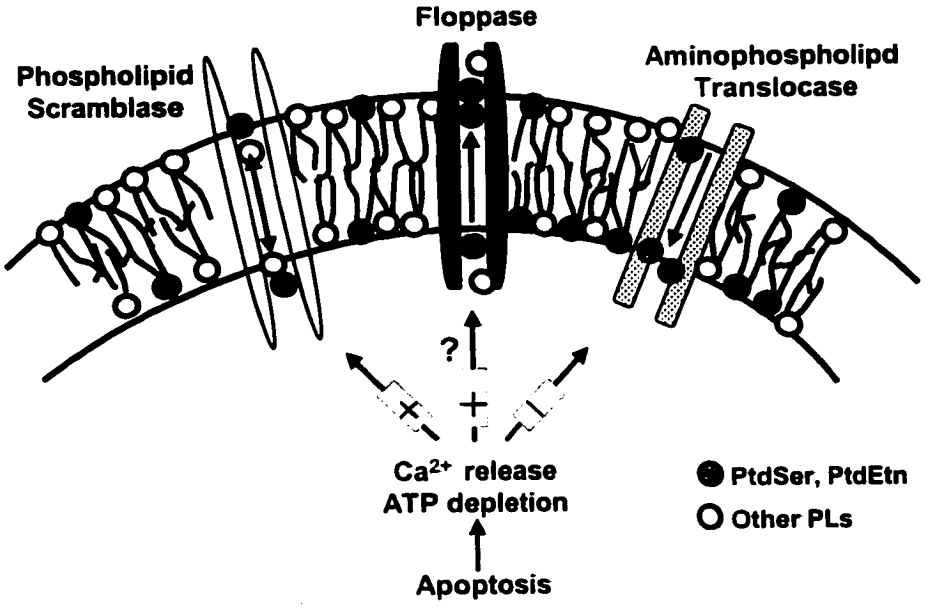

Figure 6. Proposed lipid transporters on the plasma membrane. Three lipid transporters exist on the plasma membrane and are responsible for the establishment and maintenance of lipid asymmetry under normal conditions. Collapse of lipid asymmetry also results from the differential regulation of these lipid transporters. 1. Aminophospholipid translocase (APT) is an ATP-dependent transporter specifically moving exposed PtdSer and PtdEtn inward, thus confining them to the inner layer. APT activity is inhibited by depletion of ATP and high levels of cytosolic Ca^{2+} during apoptosis. 2. Floppases are a group of outward lipid transporters. They translocate phospholipids at a slow rate to the cell surface at the expense of ATP. 3. Phospholipid scramblase (PLSCR) is a Ca^{2+}-stimulated lipid transporter for all phospholipids. Its activation during apoptosis results in the redistribution of phospholipids and externalization of PtdSer. The illustration is adapted from a similar one (Diaz and Schroit, 1996).

predominates. As a result, when PtdSer and PtdEtn are flipped spontaneously or by facilitation to the cell surface, they will be translocated back into the inner leaflet of the plasma membrane (Bevers et al., 1996).

Membrane-bound proteins with putative APT activity (PtdSer-specific, Ca^{2+}-, sulfhydryl- and vanadate-sensitive Mg^{2+}-ATPase) have been purified from various sources such as erythrocytes, chromaffin granules and synaptic (clathrin-coated) vesicles (Morrot et al., 1990; Wang et al., 1988; Hicks and Parsons, 1992). A bovine gene encoding 1148 amino acids of the putative APT protein has been cloned using RT-PCR techniques and sequence analysis has identified this protein as a novel subfamily member of P-type ATPase requiring Mg^{2+} for activity (Tang et al., 1996). P-type ATPases are ATP-fueled ion pumps with a single catalytic subunit and characterized by a phosphorylation reaction cycle at an aspartate residue. This putative APT differs from regular P-type ATPases in the modification of the substrate-binding site where hydrophobic residues capable of lipid binding replace negatively charged residues for cation binding. The closest homolog is *Drs2* gene in yeast (Tang et al., 1996). However, deletion of the *Drs2* gene in yeast does not result in the loss of inward transport of aminophospholipids indicating Drs2p protein is not the main APT in yeast and that another APT definitely exist (Siegmund et al., 1998). Furthermore, lack of expression of ATPase (proposed as APT) in various cells and tissues does not lead to changes in membrane asymmetry (Mouro et al., 1999). Four isoforms of P-type ATPase with different ATPase activities in response to phospholipids species now have been identified in bovine brain as possible candidates for APT. To varying degrees, all require PtdSer for dephosphorylation to complete the catalytic cycle (Ding et al., 2000). These data indicate

that multiple potential candidates of APT may function under different conditions in various types of cells; alternatively, multiple components contribute to or regulate the activity of APT (Daleke and Lyles, 2000). More work is needed to reveal the true identity and control mechanisms of APT.

b. Floppase

In human erythrocytes, the activity of a non-specific outward lipid transporter or "floppase" has also been reported (Bitbol and Devaux, 1988). This transporter has a half-life of 1.5 h which is much greater than the 5-10 min half-life of APT (Bevers et al., 1999). ATP-binding cassette (ABC) transporters represent a large family of active transporters that bind and move various substrates through the membrane against a concentration gradient by using energy from ATP hydrolysis. Several members of the ABC transporters, including multidrug resistance protein 1 (MRP1), MDR3 in humans and ATP-binding cassette transporter 1 (ABC1), are candidates with putative floppase activities involving translocation of distinct types of lipids to the cell surface at the expense of ATP (Borst et al., 2000; Raggers et al., 2000).

In human erythrocytes, outward movement of NBD-labeled PtdSer or PtdCho needs the coexistence of ATP and oxidized glutathione (GSSG) and is blocked by inhibitors of MRP1. Floppase has been suggested to be MRP1, also known as glutathione-conjugated transporter (GS-transporter) (Kamp and Haest, 1998). Erythrocytes from MRP1 knockout mice show a complete defect in outward movement of the fluorescent phospholipid analogues (Dekkers et al., 1998a). Prolonged inhibition of MRP1, without affecting APT activity, results in a decreased distribution of endogenous PtdCho and SM on the outer layer of the plasma membrane of human erythrocytes whereas asymmetric distribution of

amino-phospholipids is not affected (Dekkers et al., 2000). It has been suggested that enrichment of choline-phospholipids on the cell surface may be not only a passive outcome of the inward movement of aminophospholipids by translocase, but also could be actively promoted by MRP1 through the concerted outward movement of choline-containing phospholipids.

Other members of this MRP family also have been reported as lipid transporters in various cell systems (Raggers et al., 2000; Ruetz and Gros, 1994). A P-glycoprotein, encoded by *mdr2* gene in mice or *MDR3* gene in humans, is identified as a PtdCho-specific floppase and is essential for the secretion of PtdCho into bile in liver (Smit et al., 1993; Smith et al., 1994). An unidentified P-glycoprotein can move SM outward in leukemic cells and may be responsible for enrichment of SM in the exoplasmic face of the plasma membrane (Bezombes et al., 1998).

ABC1 protein has been found to be mutated in patients with Tangier disease. These patients have disorders in lipid metabolism and show massive accumulation of cholesterol-esters in various tissues. ABC1 was initially proposed to be a cholesterol transporter responsible for the cellular export of cholesterol (Orso et al., 2000). Interestingly, ABC1 is homologue to the product of *C. elegans* gene *ced7* that functions in the engulfment of cell corpses during programmed cell death (Wu and Horvitz, 1998). Engulfment of apoptotic thymocytes by macrophages is inhibited by an ABC1 blocker, glyburide, through the impairment of PtdSer externalization on both the prey and the phagocytes (Marguet et al., 1999). Further loss or gain of function studies prove that the ABC1 protein can promote Ca^{2+}-stimulated externalization of PtdSer and consequently the engulfment of apoptotic cells. It is suggested that efflux of cholesterol and choline

phospholipids to specific receptors requires the transbilayer redistribution of PtdSer and subsequent membrane vesiculation promoted by ABC1 (Hamon et al., 2000). Thus, it is likely that ABC1 is a PtdSer-specific floppase that indirectly mediates efflux of cholesterol and other lipids through formation of microvesicles mediated by PtdSer externalization.

Many other members of this ABC transporter family have the ability to transport lipids along with other chemicals (Borst et al., 2000). Thus, the real picture of this so-called floppase is far from clear and needs further investigation.

c. Phospholipid scramblase

While establishment of lipid asymmetry depends on the activities of aminophospholipid translocases and floppases, changes in the normal asymmetrical equilibrium require the active role of a bi-directional lipid transporter termed phospholipid scramblase (PLSCR). In human erythrocytes, elevation in cytosolic calcium levels results in a rapid diffusion of spin- or fluorescent-labeled phospholipids analogues of all major species across the plasma membrane (Williamson et al., 1992). This Ca^{2+}-stimulated lipid scrambling is bidirectional and involves all major phospholipids with lower preference toward SM (Smeets et al., 1994). Using NBD-PtdSer as a probe, a continuous flop of internalized NBD-PtdSer to the outer membrane surface was observed in platelets activated with calcium ionophores or other stimuli. This scrambling activity is sensitive to the sulfhydryl reagent suggesting the involvement of a thiol-dependent protein in the process (Williamson et al., 1995).

A 35 kDa membrane protein, isolated from human erythrocytes using anion exchange chromatography and reconstituted into proteoliposomes, showed phospholipid

scramblase activity that moved NBD-PtdSer in and out of lipid bilayers. This process is stimulated by Ca^{2+} but not Mg^{2+} (Basse et al., 1996). Molecular cloning of this human phospholipid scramblase (later identified as HuPLSCR1) revealed a proline-rich, type II transmembrane protein encoded by a 1,445 bp cDNA. Screening for PLSCR mRNA showed universal expression in a variety of human tissues and cell lines except for Raji and MOLT-4 cells (Zhou et al., 1997). When EGFP-conjugated PLSCR was over-expressed in Raji cells that are low in endogenous PLSCR, PtdSer externalization to the cell surface increased following calcium ionophore treatment providing the first evidence for its role in transbilayer lipid movement (Zhao et al., 1998a).

A family of phospholipid scramblases, including HuPLSCR 1-4 and their murine orthologues, MuPLSCR 1-4, as well as a rat PLSCR homolog to HuPLSCR1, has been identified (Wiedmer et al., 2000) (Fig. 7). PLSCR genes are well conserved through evolution as putative PLSCR homologues also are identified from yeast (Jelinsky et al., 2000). PLSCR proteins have a short or absent extracellular domain whereas their intracellular domains are highly variable in length and composition (Pastorelli et al., 2001). Expression of PLSCR1 is induced at the transcriptional level by interferon (IFN) indicating its potential involvement in IFN-mediated activities (Zhou et al., 2000). The PLSCRs are type II transmembrane proteins that are highly conserved in the calcium-binding C-terminal domain (Zhou et al., 1998). Calcium binding to the proteins is presumed to induce a major conformational change and activate the lipid scrambling activity (Stout et al., 1998). PLSCR modifications have been implicated in regulating its normal functions. Palmitoylation at multiple cysteine residues on the protein contributes to the membrane anchoring and normal functions of PLSCR (Zhao et al., 1998b). Attachment of scramblase polypeptide to the plasma membrane through palmitoyl chains

may play an important role in mediating Ca^{2+}-dependent activity of PLSCR (Zhao et al., 1998b).

Some members contain Pro-XX-Pro and Pro-Pro-X-Tyr motifs at N-terminus indicating potential interaction with signaling molecules that contain SH3 or WW domains (Sims and Wiedmer, 2001; Wiedmer et al., 2000). PLSCR1 is a substrate of several kinases including PKC δ, c-Abl, IgE-coupled kinase and EGF receptor kinase. PLSCR1 binds to the SH3 domain of c-Abl tyrosine kinase and is constitutively tyrosine phosphorylated by this enzyme (Sun et al., 2001) at two tyrosine residues within a region unique to human PLSCR1 (Fig. 7). The interaction between c-Abl and human PLSCR1 may contribute to the cellular response to genotoxic stress. Phosphorylation of human PLSCR1 by PKC δ at Thr-161 is observed during apoptosis and cell activation and the modification of PLSCR1 protein is crucial for the induction of PtdSer exposure in response to apoptotic stimuli (Frasch et al., 2000). Epidermal growth factor (EGF) stimulates cellular expression of PLSCR1 and triggers c-Abl-independent tyrosine phosphorylation of PLSCR1 through unidentified EGF receptor kinase. PLSCR1 and EGF receptor are enriched in lipid rafts and a transient physical interaction between PLSCR1, EGF receptor and adaptor protein Shc occurs following EGF stimulation (Sun et al., 2002). PLSCR also may be involved in signaling by an immune receptor as rat PLSCR is phosphorylated by IgE-coupled tyrosine kinase activity (Pastorelli et al., 2001). Thus, PLSCR proteins may not only serve to activate membrane lipid scrambling, but also play versatile roles in signaling pathways that regulate cellular events such as immune responses, responses to stress, proliferation, differentiation and oncogenic transformation (Sun et al., 2002; Silverman et al., 2002; Zhou et al., 2002). The actual functions of members of PLSCR family remain largely unknown and await further investigation.

Figure 7. Multiple alignment of protein sequences of members of phospholipid scramblase family. Predicted protein sequences of HuPLSCR1 (GenBank accession number NP_066928), MuPLSCR1 (GenBank accession number AAF77076), and MuPLSCR2 (GenBank accession number AAC40053) were aligned using CLUSTALW. Identical amino acids are shown in outlined boxes and conservative substitution are on a gray background. Conserved Ca^{2+}-binding and transmembrane (TM) domains are located at the C-terminus of the proteins. The N-terminal region is rich in proline residues and may mediate binding with proteins containing SH3 or WW domains. Tyrosine phosphorylation catalyzed by cAb-1 kinase occurs at a region unique to human PLSCR1. PKC δ phosphorylates human PLSCR1 at Thr-161 and sequences surrounding this residue are highly conserved among different PLSCR isoforms. Multiple cysteine residues may be palmitoylated to anchor polypeptide toward the plasma membrane. Disulfide bonds also may form as the scramblase is reported to be a thiol-dependent protein.

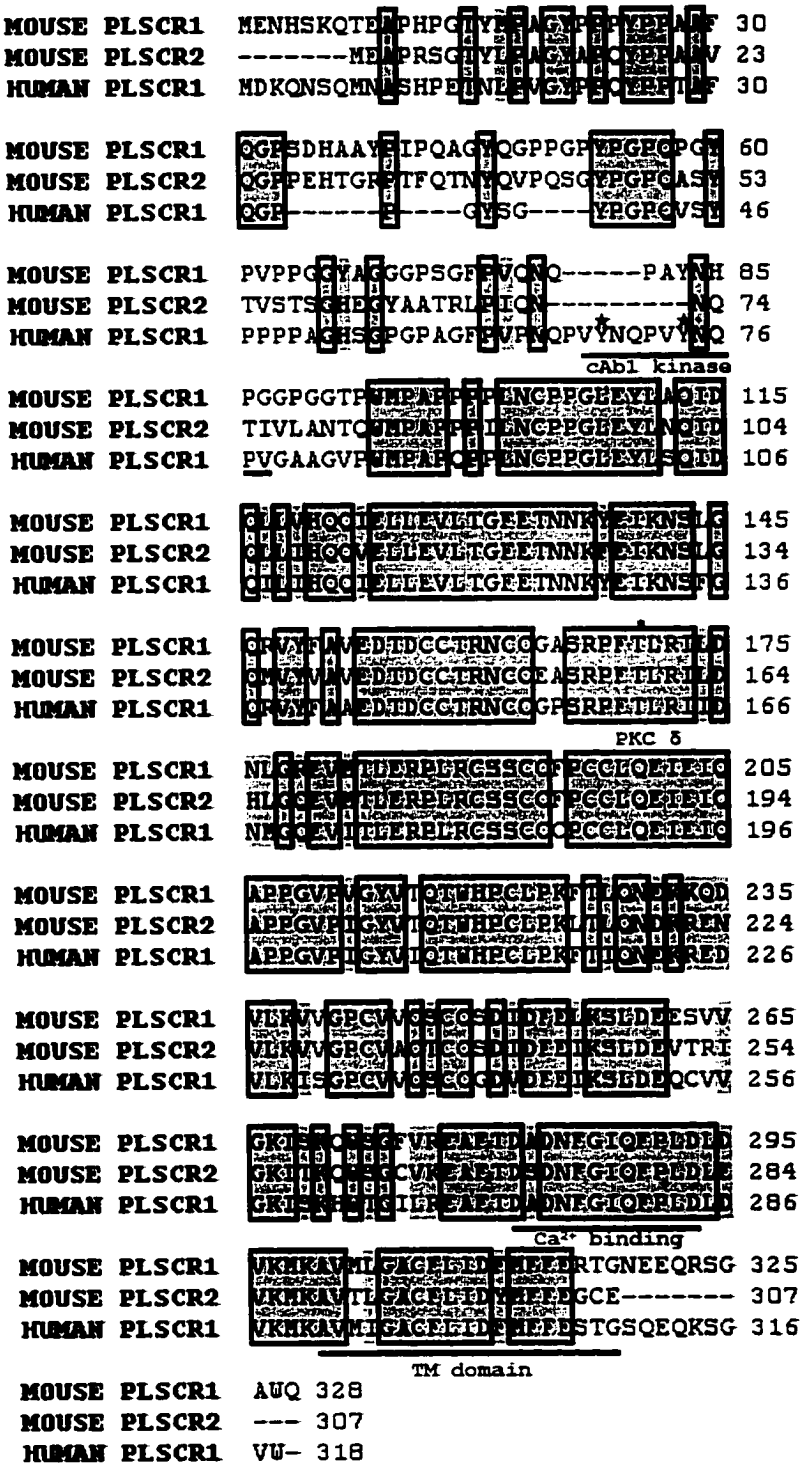
MOUSE PLSCR1
MOUSE PLSCR2
HUMAN PLSCR1
cAbl kinase
PKC δ
Ca++ binding
TM domain

Figure 7

Plasma membrane asymmetry partly results from the spatial segregation of phospholipid biosynthesis; for example, PtdSer synthesized on the cytoplasmic surface of ER is destined to go to the inner leaflet of the plasma membrane. Membrane asymmetry then is established and maintained by the concerted action of APT and floppase. APT plays a surveillance function by transporting exposed amino-phospholipids inward rapidly and simultaneously. The floppase mediates the outward transport of phospholipids to replace aminophospholipids. Because activity of APT is much higher than that of the floppase, confinement of aminophospholipids to the inner layer of plasma membrane is the net result of this dynamic process. Maintenance of membrane asymmetry is also safeguarded by the inactivation of PLSCR activity due to low intracellular concentrations of calcium in quiescent cells.

2. Loss of membrane asymmetry and its physiological significance

Although maintenance of membrane asymmetry is vital to ensure normal functions of mammalian cells, its collapse also plays crucial roles in signaling major shifts in the physiological state of the cells. Appearance of PtdSer on the cell surface as a result of collapse of membrane is observed in activated (Bevers et al., 1982), aging (Connor et al., 1994) and apoptotic cells (Martin et al., 1995a).

a. Collapse of lipid asymmetry

Aminophospholipid translocase is the key enzyme in maintaining lipid asymmetry because it brings PtdSer and PtdEtn that are flipped spontaneously outside to the inner layer. APT is much more active than the floppase activity and thus most of the aminophospholipids are confined to the inner layer of the plasma membrane. APT is a Mg^{2+}-ATPase that needs ATP and PtdSer for activity but is inhibited by Ca^{2+}. As a result, cellular events that result in ATP depletion or elevation of cytosolic Ca^{2+} levels may lead to inhibition of APT activity. Floppase also requires ATP hydrolysis for its transport

activity. Increases in cellular Ca^{2+} levels are a general response to various stimuli during cell activation and programmed cell death (McConkey and Orrenius, 1996a; McConkey and Orrenius, 1996b). In contrast, apoptotic and senescent cells have a lower capacity for producing ATP (Bossy-Wetzel et al., 1998; Vander Heiden et al., 1999) and consequently the inhibition of APT activity may contribute to PtdSer externalization (Fig. 6). Earlier studies using apoptotic thymocytes and lymphocytes showed loss of APT in a time dependent manner (Verhoven et al., 1995). Subsequent work provided evidence that loss of APT activity alone is insufficient for loss of lipid asymmetry (Bratton et al., 1997), but loss of APT enhances PtdSer externalization, while Ca^{2+}-mediated lipid flip-flop catalyzed by PLSCR is the primary force driving the randomization of membrane lipids (Bratton et al., 1997).

b. Physiological roles of loss of membrane asymmetry

PtdSer externalization is one of the hall marks of apoptotic cells and plays important roles in mediating the removal of dead cells (Fadok and Chimini, 2001). Other physiological roles also have been suggested as PtdSer externalization occurs in many cellular events.

(1) Homeostasis and thrombosis

PtdSer externalization is important in the removal of senescent blood cells. Human red blood cells (RBCs) have a short half-life and the increasing age of RBCs is accompanied by increased PtdSer on the outer leaflet of the plasma membrane. This signals removal of old or damaged cells by macrophages (Connor et al., 1994). Thus, normal turnover of RBCs can be maintained even without induction of apoptosis in senescent RBCs (Zwaal and Schroit, 1997).

Exposed PtdSer is the assembly centre for tenase and prothrombinase complexes that consist of a variety of protein factors mediating the blood clotting process. PtdSer also regulates coagulation both positively and negatively to achieve the balance in formation of blood clots (Bevers et al., 1982; Schroit and Zwaal, 1991).

(2) Myoblast differentiation

PtdSer is exposed on the surface of muscle cells and membrane areas with exposed PtdSer tend to form protrusions. As a result, these cell surface extensions become the sites for contact between muscle cells. This is essential for the formation of myotubes during differentiation of muscle cells (van den Eijnde et al., 2001).

(3) Related diseases

Malfunctions in the regulation of PtdSer externalization result in several human diseases. Scott syndrome is one such rare recessive genetic disease. RBCs from Scott syndrome patients cannot externalize PtdSer and as a result, these patients develop severe bleeding disorders (Dekkers et al., 1998b). The presence of oligonucleosomes in the blood from SLE patients indicates a failure in clearance of apoptotic cells. Mechanisms underlying the defects in this disorder are still unclear but are associated with disturbances in recognizing apoptotic cells with exposed PtdSer (Fadeel et al., 1999a).

3. PtdSer exposure as a feature of apoptotic cells

Early connections between loss of lipid asymmetry and programmed cell death arose from studies on removal of apoptotic blood cells by macrophages. Membrane lipid composition was proposed to determine the interaction of blood cells and reticuloendothelial cells (Schlegel and Williamson, 1987). Eventually PtdSer exposure was reported in apoptotic blood cells of human and murine origin (Fadok et al., 1992a;

Fadok et al., 1992b) and was shown to be involved in the initiation of macrophage recognition. PtdSer externalization was identified as a general feature of apoptosis among many types of cells regardless of the initiating stimuli and was shown to happen at an early stage of cell death (Martin et al., 1995a). Subsequent studies have revealed that PtdSer externalization during apoptosis occurs in a variety of mammalian cells ranging from smooth muscle to spermatogenic cells (Schlegel and Williamson, 2001). Furthermore, PtdSer exposure has been observed in apoptotic cells from ectoderm, endoderm and mesoderm of chicken embryos and *Drosophila* pupae indicating that this phenomenon is phylogenetically conserved in cells from all lineages (van den Eijnde et al., 1998). Genes regulating PtdSer exposure and removal of apoptotic cells are conserved from *C. elegans* to mammalian cells (Henson et al., 2001a). Thus, PtdSer externalization is widely considered as a general biochemical change during programmed cell death and can be used as a marker to identify apoptotic cells.

a. Detection of PtdSer externalization

Detection of PtdSer expression at the cell surface is an important tool in identifying apoptosis and understanding its regulation and physiological implications. One early method indirectly monitored PtdSer appearance by measuring the procoagulant activity exhibited by the cells in the presence of Russell's viper venom (Chiu et al., 1981). More direct detection of surface PtdSer comes from identification of annexins, a family of phospholipid binding proteins (Raynal and Pollard, 1994). Annexin-V, initially isolated from human arteries as a novel anticoagulant (Reutelingsperger et al., 1985; Reutelingsperger and van Heerde, 1997), binds to PtdSer in a Ca^{2+}-dependent manner (Meers and Mealy, 1993; Tait and Gibson, 1992). FITC-conjugated annexin-V has been

successfully used in the screening of apoptotic human cells, providing evidence that PtdSer externalization is a general feature of apoptosis (Zhang et al., 1997). Annexin-V, in combination with propidium iodide (PI) to asses the membrane integrity of cells based on exclusion of this cell-impermeable nuclear dye, has been used in many studies as an efficient and specific assay to detect apoptosis based on surface exposure of PtdSer (van Engeland et al., 1996; van Engeland et al., 1998). Normal cells are identified as annexin-V and PI negative, necrotic cells or late apoptotic cells as annexin-V and PI positive, and cells dying by apoptosis as annexin-V positive and PI negative. Annexin-V can also be administered to living organisms to detect PtdSer exposure *in vivo* (Blankenberg et al., 1998). Development of this convenient assay has provided a powerful tool for research into the mechanisms of both programmed cell death and loss of lipid asymmetry. However, evidence that annexin-V also binds with membrane products of lipid peroxidation casts doubts on whether annexin-V is a specific probe for detection of exposed PtdSer (Balasubramanian et al., 2001).

b. Regulation of PtdSer exposure during apoptosis

(1) Lipid transporters exposing PtdSer in apoptotic cells

Apoptotic cells provide good models for studying the mechanisms of redistribution of membrane lipids, as down-regulation of APT and up-regulation of PLSCR are considered the main contributors to PtdSer externalization during apoptosis. Apoptotic signaling pathways control the initiation of PtdSer externalization with mechanisms differing from those involved in regulation of PtdSer externalization in cell activation.

PtdSer externalization observed in dying cells is a prolonged process. Phospholipid scramblase can be up-regulated using different mechanisms under various circumstances

(Williamson et al., 2001; Williamson et al., 1995; Kamp et al., 2001). When Jurkat cells are activated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), PLSCR is activated transiently, usually resulting in a short appearance of PtdSer on the cell surface. Because aminophospholipid translocase is still active in these stimulated cells, some redistributed PtdSer resulting from transient activation of PLSCR will be moved back to the inner membrane (Frasch et al., 2000). However, when apoptosis is initiated in Jurkat cells using anti-Fas antibody, activation of scramblase activity results in sustained and maximum PtdSer externalization in the apoptotic cells. PLSCR may be differentially regulated by PKCδ. In signaling pathways resulting in cell activation, PKCδ translocates to the plasma membrane where it can phosphorylate a threonine residue of scramblase. Under Fas-mediated apoptosis, PKCδ is irreversibly activated through caspase-3 cleavage and release of the active catalytic domain. This constitutively active PKCδ may up-regulate scramblase in a prolonged manner (Frasch et al., 2000).

As cleavage of protein components is a general consequence of the activation of caspases, a role for fodrin, a cytoskeletal protein, has been proposed in regulating PtdSer redistribution in apoptotic cells. Under normal circumstances, fodrin binds PtdSer in the inner leaflet of the plasma membrane and anchors PtdSer to keep membrane asymmetry (Manno et al., 2002). Fodrin cleavage and PtdSer externalization occur simultaneously during Fas-mediated apoptosis in Jurkat cells (Vanags et al., 1996), but there is no convincing evidence for a direct cause and effect relationships between these two events.

Oxidation of PtdSer in apoptotic cells has implications in regulating PtdSer externalization. In several cell lines, PtdSer in the plasma membrane is preferentially oxidized during apoptosis mediated by oxidative stress (Fabisiak et al., 1998; Schor et al.,

1999; Tyurina et al., 2000). Oxidation of PtdSer precedes its appearance at the cell surface and this modification of PtdSer may facilitate externalization. Oxidized phospholipids undergo spontaneous flip-flop more easily than is the case for their non-oxidized counterparts. Also, oxidized PtdSer directly inhibits APT activity (Herrmann and Devaux, 1990). Excessive cytochrome C is released into the cytoplasm during apoptosis where only a few molecules are needed for catalyzing the formation of apoptosomes. When Bcl-2 was over-expressed in several cell lines, blockage of cytochrome C release was accompanied by prevention of PtdSer oxidation (Fabisiak et al., 1997). One possibility is that cytochrome C oxidizes PtdSer in the inner face of the plasma membrane and accelerates the exposure of PtdSer on the outer membrane surface (Kagan et al., 2000). Overall, it appears that PtdSer oxidation is sufficient, but not necessary, for APT inhibition and PtdSer externalization during apoptosis.

(2) Roles of caspases in PtdSer externalization

Caspases are key players of programmed cell death. Initiation and effector caspases are sequentially activated in the apoptotic process and these activities are implicated in regulating PtdSer externalization (Schlegel and Williamson, 2001). Evidence for PtdSer externalization as a downstream event mediated by caspase activation comes from the use of caspase inhibitors, such as z-VAD-fmk. When applied to cells, z-VAD-fmk is able to block cleavage of effector caspases such as caspase-3 and this is accompanied by blockage of PtdSer externalization and other apoptotic changes in a concentration-dependent manner (Schnaper, 2000) (Naito et al., 1997). z-VAD-fmk is a potent, broad-range inhibitor with different levels of efficiencies on at least 10 caspases (Garcia-Calvo et al., 1998). Earlier studies indicated that Bcl-2 protein, an anti-apoptotic protein, had a

negative role in regulating PtdSer exposure; over-expression of Bcl-2 inhibited PtdSer externalization as well as apoptosis in CEM cells (Fabisiak et al., 1997). Embryonic stem cells or fibroblasts from Apaf1 knockout mice showed defects in exposure of PtdSer on the cell surface following treatment with a variety of death stimuli indicating that PtdSer externalization is a downstream event of apoptosome formation and activation of caspase-9 (Yoshida et al., 1998). PtdSer externalization and caspase-3 activation were uncoupled during TNF-α-induced apoptosis in U937 cells using inhibitors of mitochondrial functions such as antimycin D and oligomycin that block the respiratory chain and ATPase, respectively (Zhuang et al., 1998). PtdSer externalization can be impaired in other cases by inhibitors of caspase-3 (Mandal et al., 2002; Martin et al., 1996). There are also indications of caspase-independent PtdSer externalization (Huigsloot et al., 2001; Martinez and Freyssinet, 2001; Mann et al., 2000). When chicken erythrocytes and mouse sperm that do not have active nuclei were investigated, cell death occurred without activation of caspases, whereas annexin-V assays revealed PtdSer externalization on the outer leaflets of the cells (Weil et al., 1998).

Calpain-mediated cleavage of cytoskeletal proteins is involved in the loss of lipid asymmetry during activation of platelets through calcium signaling (Comfurius et al., 1990). Calpain is a protease that mediates some forms of cell death without the involvement of caspases (Johnson, 2000). Calpain may destabilize membrane asymmetry and induce PtdSer externalization during apoptosis through cleavage of cytoskeletal protein such as fodrin (Vanags et al., 1996). Whether PtdSer is downstream of calpain activation in these cases needs further investigation.

In conclusion, PtdSer externalization is an important event and marker of programmed cell death and is universally present in all forms of cell death. PtdSer externalization during apoptosis is generally under the control of the death program. However, the regulatory mechanisms underlying transbilayer redistribution of PtdSer vary depending on the types of cells and the apoptotic stimuli involved. It is likely that PtdSer externalization is a downstream event of initiator caspases but may be independent of effector caspases in some cell types. In cases of cell death that are independent of caspases, PtdSer can still be exposed but its initiation needs further study.

c. Roles of PtdSer externalization during apoptosis

(1) Removal of apoptotic cells by macrophages

One of the key differences between apoptosis and necrosis is that cells undergoing apoptosis express membrane signals at the surface that initiate recognition by macrophages. Engulfment and digestion of apoptotic cells prevent the release of harmful contents from dead cells and avoid the induction of inflammatory responses. In contrast, necrotic cells lose membrane integrity and release their cellular contents, triggering inflammatory responses in the host organism. Thus, recognition and removal of apoptotic cells have significant physiological roles in organism development and normal cell turnover of multi-cellular organisms. PtdSer is one of the key signals expressed at the surface by apoptotic cells. As normal cells do not have external PtdSer at the plasma membrane, PtdSer serves as a surface marker for apoptotic cells (Fadok et al., 1992b; Aderem and Underhill, 1999). Several membrane-associated or soluble proteins have the ability to bind PtdSer and to mediate phagocytosis by macrophages.

β_2-glycoprotein I (β_2GPI) is a serum protein that binds to PtdSer liposomes *in vitro*. Association of β_2GPI with PtdSer of apoptotic thymocytes or red blood cells helps their removal from the circulation by macrophages, playing an important role in regulating homeostasis (Balasubramanian et al., 1997). Conformational changes of β_2GPI induced by PtdSer generate a binding moiety that enables the interaction with unidentified receptors on macrophages (Balasubramanian and Schroit, 1998). Thus, β_2GPI serves as an intermediate bridge between PtdSer expressing cells and macrophages and directs the removal of cells with externalized PtdSer.

Class B scavenger receptors, such as class B scavenger receptor type I (SR-BI) and CD36, are another group of proteins capable of binding to anionic phospholipids (Rigotti et al., 1995). Maturation of spermatogenic cells is accompanied by PtdSer externalization before they are engulfed by Sertoli cells. SR-BI in testis is considered to be a strong candidate for a PtdSer receptor. Over-expression of SR-BI in Sertoli cells increases PtdSer-dependent phagocytosis of apoptotic spermatogenic cells and this can be blocked by the presence of PtdSer. Antibodies of SR-BI inhibit phagocytosis to an extent similar to PtdSer liposomes. Thus, SR-BI acts as one of the PtdSer receptors of Sertoli cells with an ability to induce phagocytosis (Shiratsuchi et al., 1999). Binding of vesicles containing PtdSer to the monocytic leukemia cells was blocked up to 60% by a monoclonal antibody to CD36 (Tait and Smith, 1999). CD36 appears to function partially as a PtdSer receptor in these cells mediating the binding of anionic phospholipid vesicles.

Protein encoded by the growth arrest-specific gene 6 (Gas 6) is a soluble protein secreted by vascular smooth muscle cells (Manfioletti et al., 1993; Nakano et al., 1995) and serves as ligand of the Axl/Mer/Tyro3 tyrosine receptor family (Nagata et al., 1996).

Gas 6 specifically binds to PtdSer showing no recognition of other major phospholipids, including PtdEtn. Gas 6 can only bind to Axl-expressing U937 cells in the presence of PtdSer (Nakano et al., 1997). As Gas 6 initiates receptor tyrosine kinase activity, the interaction bridged between PtdSer and membrane kinases by Gas 6 may lead to novel signal transduction pathways. One speculation is that Gas 6 recognizes PtdSer expressing apoptotic cells and directs them to macrophages with Gas 6 specific receptors such as MER, a member of the Axl/Mer/Tyro3 tyrosine receptor family. *mer*^{kd} mice with a cytoplasmic truncation of MER had macrophages deficient in the clearance of apoptotic thymocytes indicating a function of MER in regulating specific removal of apoptotic cells by phagocytosis (Scott et al., 2001; Henson et al., 2001a). Thus, PtdSer serves not only as a marker of apoptosis, but also as a trigger of signaling events that helps the clearance of apoptotic cells.

A novel receptor for PtdSer, identified as PtdSer receptor (PSR), has been cloned (Fadok et al., 2000). Two monoclonal antibodies (mAb217 and mAb284) that are highly stimulated in human macrophages induced to recognize PtdSer have been generated. Phage display biopanning techniques have been used to identify the antigen for mAb217 as a 47~48 kDa transmembrane protein. Transfection of Jurkat cells with the putative PtdSer receptor confers PtdSer-specific binding and removal of apoptotic lymphocytes, a process that does not occur under normal circumstances. When lipopolysaccharide-induced Jurkat cells that stably express PSR were treated with PtdSer liposomes or mAb217, down-regulation of TNF-α and up-regulation of TNF-β were observed. As TNF-α is a pro-inflammatory molecule and TNF-β functions in the opposite way, PSR may also mediate PtdSer-dependent down-regulation of inflammatory responses by

facilitating removal of apoptotic cells. PSR is universally expressed and conserved through evolution as its homologue has been identified in *C. elegans* and *Drosophila.*

Since multiple proteins are involved in PtdSer-dependent or -independent binding and removal of apoptotic cells, it is difficult to study the specific role of an individual component. A novel technique for testing the roles of different protein ligands in mediating recognition and removal of apoptotic cells has been developed. Briefly, the surface proteins of human erythrocytes were biotinylated and coated with avidin. These erythocytes were then bound to a biotinylated ligand or antibody for the desired receptor to form surrogates of apoptotic cells that can bind to macrophages through the activity of a specific protein ligand (Hoffmann et al., 2001). Many proposed phagocytic receptors, such as CD36, class A scavenger receptor or vitronectin $\alpha_v\beta_5$, when tested with these surrogate cells in the absence of PtdSer, promote tethering but not engulfment by macrophages; addition of PtdSer leads to the engulfment of cells coated with the ligands. This PtdSer-dependent engulfment requires PSR because it can be inhibited by anti-PSR antibody or anti-sense expression of PSR. Both anti-PSR antibody and PtdSer stimulate membrane ruffling and macro-pinocytosis, implying that binding of PtdSer by PSR may trigger membrane ruffling needed in phagocytosis. Thus, a "tethering and tickling" mechanism has been proposed, suggesting that multiple ligands (PtdSer, modified sugar residues or other moieties on the surface of apoptotic cells) exist as tethering signals that regulate the binding of apoptotic cells to macrophages. Tethering results in close contact between PtdSer and PSR at the surface of macrophages and subsequently initiates membrane ruffling and uptake of apoptotic cells through PSR-mediated signaling. Stimulated PSR also inhibits initiation of inflammatory responses and blocks the

maturation of dendritic cells and the subsequent immune responses (Henson et al., 2001b). Thus, PSR is suggested as a crucial "molecular switch" playing versatile roles in regulating inflammation and immunity during clearance of both apoptotic and necrotic cells. Studies with cystic fibrosis indicated that PSR is subjected to cleavage by elastase secreted by neutrophils. This results in disruption of phagocytosis of apoptotic cells and may be the main reason for accumulation of apoptotic cells inside the patient's airways and for elevation of inflammatory responses (Vandivier et al., 2002).

(2) Phospholipid scrambling-regulated membrane vesiculation

PtdSer externalization serves mainly as a marker for apoptotic cells to initiate phagocytosis, anti-inflammatory reactions and immuno-suppressing responses through activation of PSR. Another role for PtdSer externalization during apoptosis is the regulation of membrane vesiculation. Maintenance of lipid asymmetry in mammalian cells stabilizes membrane structure and interactions with cytoskeleton proteins (Manno et al., 2002). Randomization of PtdSer on the cell surface seems to promote membrane protrusions required in the formation of myotubes during development of muscles (van den Eijnde et al., 2001). Similarly, PtdSer externalization contributes to extensive membrane invagination and formation of vesicles during apoptosis. Surface expressed PtdSer is localized to specific membrane domains, especially membrane blebs (Walkey et al., 1996). It is not clear whether membrane vesiculation is required for PtdSer externalization or is a direct consequence of PtdSer externalization. One theory hypothesized that outward movement of PtdSer is accompanied by inward translocation of SM. As internalized SM gains access to sphingomyelinase on the inner layer of the membrane, ceramide is produced through SM hydrolysis and is thought to promote

membrane blebbing through unknown mechanisms (Tepper et al., 2000). Thus, disturbances of lipid asymmetry at the plasma membrane change the physical properties of the cell surface and may lead to formation of membrane vesicles and protrusions.

D. Relationships between PtdSer externalization and biosynthesis

PtdSer, an indispensable phospholipid for cell growth, is maintained at a relatively constant level by mammalian cells. Changes in PtdSer biosynthesis have been implicated in only a few active cellular events such as acute phase response (APR) (Vincent et al., 2001) and apoptosis (Aussel et al., 1998).

APR occurs when homeostasis of organisms is disturbed by a variety of inflammatory stimuli such as interleukins or endotoxins (Kushner and Mackiewicz, 1987; Cucuianu et al., 1996). APR is represented by major changes in expression of plasma proteins and in triacylglycerol, cholesterol and sphingolipid metabolism (Hardardottir et al., 1995; Hardardottir et al., 1994). When APR is induced in rats by injection of turpentine, the major effect on phospholipids is a significant increase of PtdSer synthesis in ER membranes and ER-derived vesicles. This is concomitant with a 2-fold increase of serine base-exchange activity in liver ER membranes isolated from the treated rats. PtdSer also is preferentially targeted to ER-derived vesicles in this experimental model (Vincent et al., 2001).

PtdSer is exposed on the surface of plasma membranes during apoptosis (Schlegel and Williamson, 2001). CD95, a strong inducer of apoptosis in Jurkat cells, initiates an early and transient phase of PtdSer synthesis. When two CD95 mAbs were compared, only the one that induced apoptosis also stimulated serine incorporation into PtdSer suggesting that an enhancement of PtdSer synthesis may be specifically related to the

induction of apoptosis. Newly formed PtdSer is rapidly transported to plasma membrane and exposed on the outer leaflet and in membrane vesicles released from apoptotic cells. Although serine base-exchange reaction is stimulated by elevated Ca^{2+} concentrations in ER (Mozzi et al., 1993; Baranska, 1989), the fact that CD95 treatment does not induce changes in Ca^{2+} homeostasis rules out the possibility of Ca^{2+}-mediated stimulation of PtdSer synthases activity during this form of induced apoptosis. CD95 appears to decrease serine uptake, so it is unlikely that a rise in PtdSer formation can be explained by an increase in substrate availability. In this model of apoptosis, the increase in PtdSer biosynthesis is accompanied by a strong inhibition of PtdEtn formation through PtdSer decarboxylation. Thus, increased PtdSer content after induction of apoptosis can at least partly be explained by accumulation after an arrest of PtdSer decarboxylation in apoptotic Jurkat cells mediated through the CD95 signaling pathway.

There is evidence that serine base-exchange enzyme system also may regulate PtdSer exposure at the cell surface during CD95-induced apoptosis. All known stimulators of PtdSer biosynthesis, such as CaM antagonists, K^+-channel blockers and CADs, when applied to the CD95-treated Jurkat cells, increase the externalization of PtdSer at the cell surface. Conversely, pretreatment of the cells with CD3 mAb or thapsigargin, known for their inhibitory effects on PtdSer synthesis, markedly reduced the CD95-induced appearance of PtdSer at the cell surface (Pelassy et al., 2000a). Thus, the amount of PtdSer expressed on the outer layer of the plasma membrane during death receptor-

mediated apoptosis is at least in part regulated by the biosynthetic rate of PtdSer catalyzed by serine base-exchange enzymes.

II. Statement of Hypothesis

PtdSer appears on the outer layer of the plasma membrane during induced apoptosis in many types of cells. PtdSer externalization is considered to be a surface marker characteristic of apoptotic cells. Some studies suggest a correlation between induction of apoptosis and stimulation of PtdSer synthesis and it also appears that serine base-exchange enzyme system may regulate the externalization of PtdSer during apoptosis. Thus, we designed experiments to test the following hypothesis:

PtdSer that appears on the outer membrane surface in the early stages of apoptosis is derived from newly synthesized PtdSer and, accordingly, apoptosis may be modulated by altering PtdSer synthesis.

Experiments that form the basis of this thesis were designed to systematically test this hypothesis. To achieve this, several parameters of apoptosis that serve as markers and measures of induced apoptosis were developed and refined. Two types of cells were studied in this work. U937 cells, sensitive to many types of apoptotic stimuli, allowed extensive studies on PtdSer biosynthesis under the control of various apoptotic signaling pathways. CHO-K1 cells are susceptible to UV-induced apoptosis and enzymes catalyzing PtdSer biosynthesis or involving in scrambling of membrane phospholipids were stably over-expressed in CHO-K1 cells. These derived CHO-K1 cell lines provided tools for characterizing the relationships between new synthesis of PtdSer, the externalization of this phospholipid during apoptosis and the implications of these two events on the progression of apoptosis.

III. Materials and Methods

A. Materials

1. Cell lines

Cell lines used in this work were obtained from the American Type Culture Collection. The U937 cell line (ATCC number CRL-1593.2) was derived from malignant cells obtained from a patient with histiocytic lymphoma (Sundstrom and Nilsson, 1976). U937 cells show morphology of monocytes and can be induced to undergo terminal monocytic differentiation (Olsson et al., 1983). The cells also expressed Fas and TNF receptors on the surface and thus are sensitive to apoptosis induced by death ligands (Nilsson et al., 1981). The CHO-K1 cell line (ATCC number CCL-61) was a subclone derived from parental Chinese hamster ovary (CHO) cell line (Kao and Puck, 1968). CHO-K1 cells require proline in the medium for growth. They are adherent cells with epithelial morphology (Kao and Puck, 1967).

2. Plasmids

The pCR®2.1-TOPO® vector is part of a TOPO® TA Cloning kit from Invitrogen used for cloning PCR fragments. *Taq* DNA polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine to the 3' ends of PCR products that can then be effectively inserted into linearized pCR®2.1-TOPO® vectors that have single, overhanging 3' deoxythymidine residues. Mammalian expression vectors from Invitrogen, pcDNA3.1/Myc-His(+) version A and B, differ only in their multiple cloning sites. They contain a human cytomegalovirus (CMV) promoter that allows high levels of protein expression in most mammalian cells and a neomycin resistance gene that enables selection with G418. If properly inserted, the vectors enable the expression of

foreign proteins tagged with a Myc epitope at the C-terminus. pTK-Hyg vector is a selection vector with a hygromycin resistance gene provided by CLONTECH. When pTK-Hyg is co-transfected with expression plasmids constructed with pcDNA3.1/Myc-His(+) vector, it allows the selection of double-stable cells resistant to both G418 and hygromycin.

3. Antibodies

Several antibodies were used to detect the induction of apoptosis. Anti-human caspase-1 pAb, obtained from Upstate Biotechnology, recognizes a p20 (20 kDa) subunit of the fragmented human caspase-1 and the 45 kDa proenzyme. Anti-human caspase-3 pAb from Santa Cruz Biotechnology reacts with the p17 and p12 subunits and the 32 kDa precursor of caspase-3. Poly (ADP-ribose) polymerase (PARP) is specifically cleaved to produce a 85 kDa fragment by activated caspases such as caspase-3 (Tewari et al., 1995); anti-human PARP pAb (Santa Cruz Biotechnology) recognizes both 116 kDa PARP and the 85 kDa fragment formed after caspase-3 cleavage. Anti-ACTIVE® caspase-3 pAb, a product of Promega, is directed against a peptide from the p18 fragment of human caspase-3 and, thus, only stains apoptotic cells.

A short peptide (-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) known as the Myc epitope was added to the C-terminus of proteins for detection using Western blotting and immunofluorescence. Anti-c-Myc mAb purchased from CLONTECH recognizes the 10 amino acid epitope originally identified in human p62-c-Myc protein.

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from Bio-Rad. Fluorescein goat anti-mouse IgG,

fluorescein goat anti-rabbit IgG, and Texas Red® goat anti-mouse IgG were obtained from Molecular Probes.

4. Reagents

Most enzymes used in the molecular biology aspects of this study, including restriction enzymes, Vent® DNA polymerase and T4 DNA ligase, were purchased from New England Biolabs. *Taq* DNA polymerase was purchased from Life Technologies. A QIAprep® Miniprep Kit was from QIAGEN. The TOPO® TA cloning kit was from Invitrogen. A GENECLEAN II kit was from BIO101. All primers used were synthesized by Sigma GENOSYS. Tumor necrosis factor-α (TNF-α) was from R&D Systems. Camptothecin, cycloheximide and z-VAD-fmk were purchased from Calbiochem. Thymidine and propidium iodide were from Sigma. Radioisotopes, including L-[^{3}H(G)]serine, [methyl-^{3}H]choline chloride and [1,2-^{14}C]ethanolamine hydrochloride, were obtained from Mandel Scientific. LipofectAMINE2000 reagent, G418 and hygromycin were from Life Technologies. An annexin-V-FLUOS staining kit was from Roche Molecular Biochemicals. A Micro BCA protein kit was from Pierce. Complete™ protease inhibitor cocktail tablets were from Roche Molecular Biochemicals.

B. Methods

1. Cell culture

Human monoblastoid leukemia U937 cells were subcultured twice weekly and were cultured in a 5% CO_2 atmosphere at 37 °C in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate and 4 mM L-glutamine.

CHO-K1 cells were maintained in a 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 5% FBS and 300 μM proline.

2. Induction of apoptosis

For synchronization before induction of apoptosis with camptothecin, U937 cells were incubated at a density of 3.5 x 10^5 cells/ml in fresh growth medium supplemented with 2 mM thymidine for 16 h to induce S-phase arrest (Galavazi and Bootsma, 1966). Prior to use in experiments, cells were harvested by centrifugation at 1000 rpm for 10 min, washed twice with RPMI 1640 and resuspended in serine-free DMEM at a final density of 2 x 10^6 cells/ml. For most experiments, camptothecin was administrated at various concentrations to induce apoptosis, with or without co-incubation or pre-incubation for 1h with caspase inhibitor, z-VAD-fmk.

In some experiments, unsynchronized U937 cells were suspended at a density of 2 x 10^6 cells/ml in serine-free DMEM; TNF-α (25 ng/ml) and cycloheximide (1 μg/ml) were added or cells were exposed to UV light at 254 nm for 5 min, followed by incubation at 37 °C to induce apoptosis.

CHO-K1 cells cultured in regular growth medium were washed with and re-seeded in fresh DMEM with different modifications as indicated. Cells were exposed to a germicidal lamp providing predominantly 254-nm UV-C light (Philips TUV G30T8 30 W bulb) for 10 min and cultured for different periods of time and harvested.

3. Detection of apoptosis

a. PtdSer externalization

PtdSer exposure and membrane permeability were measured by the binding of annexin-V-FITC and propidium iodide (PI) using an annexin-V-FLUOS staining kit. After induction or inhibition of apoptosis by various treatments, U937 cells were harvested at 1000 rpm for 5 min, washed in PBS and incubated with 100 µl staining solution prepared by mixing annexin-V-FITC (1:50, v/v) and PI (1:50, v/v) in binding solution at 22 °C for 15 min according to the manufacturer's instructions. Cells were then harvested at 1000 rpm for 5 min and re-suspended in 100 µl of binding solution. Cell suspensions were mounted on glass slides and visualized by fluorescence and phase contrast microscopy using an Olympus inverted microscope model IMT2. Photographs were taken at 300X magnification. Approximately 500 cells were selected from representative fields for each sample to count cells positive for annexin-V binding.

CHO-K1 cells grown on glass cover slips were induced to undergo apoptosis with UV irradiation. Cover slips were rinsed twice with PBS and incubated with 200 µl staining solution at 22 °C for 10 min. After removal of unbound annexin-V and PI by rinsing with binding buffer, cells were fixed with 4% paraformaldehyde at 22 °C for 15 min and rinsed twice with PBS. Cover slips were mounted on glass slides. FITC 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. For the same area of each sample, a differential interference contrast (DIC) image (for total cells), a green fluorescent image (for annexin-V-positive cells), and a red fluorescent image (for PI-positive cells) were acquired; superimposed images were obtained with LSM-510 Image software.

b. Cleavage of pro-caspases and PARP

For preparations of caspase-1, caspase-3 and PARP from U937 cells for Western blotting, total protein extracts were prepared by washing cells in cold Tris-buffered saline (TBS) buffer at pH 7.5 and resuspending cell pellets in 0.5 ml lysis buffer (1% Triton-X-100, 20 µl 5 X Complete™ protease inhibitor cocktail, and 1 mM PMSF in TBS) followed by incubation on ice for 10 min. The cell lysate was centrifuged at 12,000 x *g* for 10 min at 4 °C. Protein in a 200 µl aliquot was precipitated with 1 ml cold acetone. The protein concentration of each sample was estimated using micro BCA protein assay according to the manufacturer's instructions.

For preparations of PARP from CHO-K1 cells for Western blotting, cell extracts were collected by lysing 1 x 10^7 cells in 200 µl sample buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 3.3% (v/v) β-mercaptoethanol, 12.5% glycerol, 0.05% (w/v) bromophenol blue). Cell lysates were passed through a 23G1 needle several times. The protein concentration of each sample was estimated using the micro BCA protein assay.

Samples were resolved in SDS-polyacrylamide gels (8% for PARP and 15% for caspase-1 or caspase-3) and transferred to PVDF membranes (Millipore) according to the manufacturer's instructions. For caspase-1, the membrane was blocked in 3% skim milk-PBS at 22 °C for 25 min and then incubated at 4 °C overnight with hybridization solution consisting of anti-human caspase-1 antibody (1 µg/ml) in 3% skim milk-PBS. The blot was rinsed twice with distilled H_2O and was incubated with goat, anti-rabbit, HRP-coupled secondary antibody (1:3,000, v/v) at 22 °C for 1.5 h in 3% skim milk-PBS. For PARP and caspase-3 antibody blotting, the membrane was blocked in 5% skim milk-

TTBS (0.04% Tween-20 in TBS) at 22 °C for 2 h and then incubated for 1 h with hybridization solution containing anti-human PARP (1:4,000, v/v) or anti-human caspase-3 (1:500, v/v) in 5% skim milk-TTBS. The blot was rinsed with TTBS and incubated with goat, anti-rabbit, HRP-coupled secondary antibody (1:10,000, v/v) at 22 °C for 45 min in 5% skim milk-TTBS. Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used to detect relevant proteins according to the manufacturer's protocols.

(1) Micro BCA protein assay

In some experiments, protein concentrations were measured using a Micro BCA assay kit. A working reagent was prepared by mixing Reagent MC, MB and MA (2:48:50, v/v) in that exact order. Protein samples were diluted in 0.5 ml H_2O in a 5 ml glass tube. A series of standards were made in 0.5 ml H_2O containing 0, 1, 2, 5 or 10 μg BSA/ml, respectively. Freshly made working reagent (0.5 ml) was added to standards and samples. Tubes were mixed thoroughly and placed in a hot water bath at 60 °C for 1 h. After cooling to room temperature, standards and samples were read at 562 nm using a spectrophotometer.

c. Immunofluorescence detection of caspase-3 activation

CHO-K1 cells were grown on glass cover slips. After incubation, cells were fixed with formaldehyde (3%, w/v) at 22 °C for 15 min. Cover slips were rinsed twice with 2 ml 5 mM ammonium chloride in PBS and permeabilized with 0.05% Triton-X-100 (w/v) in PBS at -20 °C for 10 min. Cells were blocked with PBS-BSA (1%, w/v) at 22 °C for 15 min. Cells were then incubated with anti-ACTIVE® caspase-3 pAb (1:500, v/v) at 4 °C overnight and rinsed twice with PBS-BSA (10 min for each rinse) at 22 °C. Cells were

further incubated with FITC-goat anti-rabbit pAb (1:500, v/v) at 22 °C for 45 min. Cover slips were rinsed twice with PBS-BSA (5 min for each time) and mounted on glass slides. FITC staining was visualized with excitation at 488 nm using a Zeiss inverted laser-scanning confocal microscope, LSM-510.

d. Nuclear morphology

For nuclear staining, U937 cells were harvested by centrifugation at 1,000 x *g* for 5 min, washed in PBS and fixed on ice with 1% glutaraldehyde (v/v) for 30 min. Cells were rinsed twice with PBS and incubated with PBS containing 100 μg/ml Hoechst 33258 (Riedel-De Haen, Germany) at 22 °C for 15 min. Staining of nuclei was visualized by fluorescence microscopy and photos were taken at 300X magnification.

CHO-K1 cells grown on glass cover slips were fixed with formaldehyde (3%, v/v) at 22 °C for 15 min. Cover slips were rinsed with 2 ml 5 mM ammonium chloride in PBS twice and permeabilized with 0.05% Triton-X-100 (w/v) in PBS at -20 °C for 10 min. Cells were blocked with PBS-BSA (1%, w/v) at 22 °C for 15 min. PI was added at a final concentration of 10 μg/ml in PBS-BSA and incubated by shaking at 22 °C for 15 min. Cells were rinsed twice with PBS-BSA (10 min each time). Cover slips were mounted on glass slides. PI staining was visualized with excitation at 543 nm using a Zeiss inverted laser-scanning confocal microscope, LSM-510.

e. Cell morphology

Phase contrast photographs of cells were taken using an Olympus inverted microscope, model IMT2, with a regular or a Nikon COOLPIX995 digital camera interphased with the microscope (usually at 300X magnification).

4. Phospholipid metabolism

a. Modified Folch lipid extraction and thin layer chromatography

Cells were rinsed and harvested into 3.5 ml ice-cold methanol:water (5:4, v/v) and transferred into glass tubes (Folch et al., 1957). Aliquots of cell suspension (100 µl) were saved in some cases for Lowry protein assay (section III.B.4.c). Chloroform (4 ml) was added to the cell suspension. Samples were mixed vigorously for 1 min on a vortex mixer and were centrifuged at 2,000 rpm for 10 min. Two fractions were separated by a thin interface of denatured protein after the centrifugation. The upper phase contains aqueous methanol and its radioactivity represents the water-soluble radioactivity incorporated. The lower phase is predominantly chloroform that dissolves the lipids and its radioactivity represents the incorporation into lipids. After removal of the upper phase into a new tube, the lower phase was washed with 2 ml ideal upper phase, a mixture of methanol, 0.9% NaCl and chloroform (48:47:3, v/v). After centrifugation, the lower phase was then aspirated into a new tube with a Pasteur pipette and saved as the lipid extract. Lipids were dried using nitrogen gas and re-dissolved in 500 µl chloroform:methanol (2:1, v/v). Radioactivity was quantitated by liquid scintillation counting using a 100 µl lipid fraction. In some case, total phospholipid mass was determined by lipid phosphorus assay using a 100 µl lipid fraction (section III.B.4.b).

Phospholipids in the lower phase were separated by thin layer chromatography (TLC). Lipids (approximately 50,000 dpm) were dissolved in a volume of 60-80 µl in chloroform:methanol (2:1, v/v). Silica gel G plates (preheated at 90 °C overnight) were prepared by drawing lines to outline lanes 1.5-2 cm in width. Lipid samples were applied in the middle of each lane and dried by nitrogen gas to generate small dots. A drop of non-radioactive phospholipid mixture from bovine brain was spotted on the sample dots and on a blank lane as a standard. Plates were developed for 2 h with 140 ml chloroform:ethanol:water:triethylamine (40:50:10:40, v/v). Organic solvent was

evaporated from the plate and it was placed on a Bioscan 200 Imaging Analyzer to identify the radioactive regions. Data were processed using Winscan software. Plates were then sprayed with phosphomolybdate spray to identify the position of individual phospholipids.

b. Lipid phosphorus assay

Total lipid mass was determined by measuring phosphorus content of the lipids (Rouser et al., 1966). Lipid samples prepared using modified Folch procedure were transferred into 10 ml glass tubes and dried with nitrogen gas. Standards of 10-100 nmol phosphorus (Na_2HPO_4) were treated similarly with samples by digestion at 120 °C overnight with perchloric acid (0.5 ml). Following cooling and addition of 3.5 ml distilled water, fresh ammonium molybdate (0.5 ml, 2.5%, w/v) and ascorbic acid (0.5 ml, 10%, w/v), samples were mixed thoroughly and heated in a hot water bath (80-90 °C) for 5 min. The blue color proportional to phosphorus mass was quantitated at 820 nm.

c. Lowry protein assay

Cell suspensions (100 µl) and standards of BSA were diluted in 900 µl 0.1 N NaOH and incubated at 4 °C overnight. Freshly prepared carbonate reagent (5 ml) was added to samples and standards and incubated at 22 °C for 10 min (Lowry et al., 1951). Diluted (1:1, v/v) Folin phenol reagent (0.5 ml) was added to samples and mixed vigorously followed by incubation at 22 °C for 30 min. Blue color proportional to protein concentration was read at 750 nm in a spectrophotometer. Protein concentrations were calculated using a standard curve plotted with Microsoft Excel program.

d. Precursor labeling

To label phospholipids in U937 cells, cells (4×10^6) were suspended in 2 ml serine-free DMEM (for serine labeling) or choline-free DMEM (for choline and ethanolamine labeling). After various treatments, cells were incubated with 6 or 12 µCi L-

[^{3}H(G)]serine/ml, 6 μCi [methyl-^{3}H]choline chloride/ml or 6 μCi [1,2-^{14}C]ethanolamine hydrochloride/ml for various times in 12-well tissue culture plates. Cells were harvested by centrifugation at 1000 rpm for 10 min and the culture medium was removed and saved. Cell pellets were rinsed twice with 2 ml cold PBS and the wash was combined with the culture medium. Cell pellets were resuspended in 2 ml methanol:water (5:4, v/v) and sonicated. Cell suspensions or medium (100 μl) was measured as described above (section III.B.4.c). Total protein of the sample was calculated as the sum of the amount recovered from cell pellets and the medium. Lipids were extracted from cell pellets and medium with the modified Folch procedure (section III.B.4.a). Radioactivity in lipid extracts was quantitated using a Beckman scintillation counter. Phospholipids were separated and quantitated using TLC. Total lipid biosynthesis was normalized with reference to total phosphorus in the lipid extracts or to total protein mass in the samples. Data from 4-8 different experiments were expressed as mean ± SEM and statistical differences were calculated using Student's *t*-test.

To label phospholipids in CHO-K1 cell lines, cells were rinsed and reseeded in serine-free DMEM (for serine labeling) or choline-free DMEM (for choline or ethanolamine labeling). After irradiation with UV light for 10 min, cell lines derived from CHO-K1 cells were incubated with 20 μCi [^{3}H]serine, 3 μCi [^{3}H]choline chloride, or 3 μCi [^{14}C]ethanolamine hydrochloride per 2 ml medium for various times. The culture medium was removed and saved. Cells were rinsed twice with 1 ml cold PBS and both washes were combined with the original culture medium. Cells were harvested; lipids were extracted and quantitated (section III.B.4.a). Data from 3-4 experiments were expressed as mean ± SEM.

e. Determination of cellular phospholipid composition

U937 cells (2 x 10^7) were seeded in 10 ml DMEM in 100 mm dishes and incubated for 4 h after administration of TNF-α and cycloheximide or exposure to UV irradiation. Cells were harvested and extracted with 4 ml chloroform (section III.B.4.a). An aliquot of lipid extract (100 µl) was used for lipid phosphorus assay. The remaining extracts were dried and re-dissolved in 80-100 µl chloroform:methanol (2:1, v/v) and were applied to a silica gel G plate. PtdCho, PtdEtn, PtdSer, SM and a total phospholipid mixture were applied to blank lanes as standards. No phospholipid mixture was over-spotted on the samples. The plates were developed in 140 ml chloroform:methanol:acetic acid:water (60:40:4:1, v/v). After evaporation of organic solvent, the plates were placed into a tank containing iodine crystals for 5-10 min. Total phosphorus in the region of each phospholipid (identified as a yellow color from iodine absorption in the sample lanes) was determined (section III.B.4.b).

5. Recombinant DNA techniques

a. Preparation of electrocompetent cells

A single clone of *E. coli*, DH5α, was inoculated into 5 ml Luria Bertani (LB) medium and was cultured at 37 °C overnight. A suspension (10 µl) of DH5α cells was transferred to 50 ml of LB medium and was cultured at 37 °C overnight. All 50 ml of bacteria culture was then inoculated into 1 liter LB medium and was incubated at 37 °C for another 2 h with shaking until the OD_{600} reached 0.6. Cells were left on ice for 10 min and pelleted by centrifugation at 5,000 rpm at 4 °C for 5 min. Cells were re-suspended in 10 ml ice-chilled 10% sterilized glycerol and 40 ml ice-chilled 10% glycerol was added and mixed by inversion. Cells were centrifuged at 5,000 rpm for 5 min and washed twice with ice-chilled 10% glycerol. After removal of the washing solution, cell pellets were re-

suspended in 1 ml ice-chilled 10% glycerol. The electrocompetent bacteria were frozen as 50 µl aliquots in liquid nitrogen and stored at -70 °C.

b. Purification of plasmid DNA

Plasmid DNA was purified with a QIAprep® Miniprep Kit based on alkaline lysis of bacterial cells followed by adsorption of DNA on silica in a high concentration of salt. DH5α™ cells with foreign plasmids were cultured at 37 °C overnight in 5 ml LB medium containing 100 µg ampicillin/ml. Bacterial cells were pelleted by centrifugation at 5,000 x *g* for 10 min and re-suspended in 250 µl buffer P1 containing RNase A. The suspension was lysed in 250 µl P2 buffer by inverting gently 4-6 times. N3 Buffer (250µl) was added to precipitate proteins and chromosomal DNA. After centrifugation at 15,000 x *g* for 10 min, the supernatant was decanted into a QIAprep spin column placed in a 2-ml collection tube. The collection tube along with the column was then centrifuged at 15,000 x *g* for 1 min; the flow-through eluted into the collection tube was discarded. The spin column was washed with 0.75 ml PE Buffer and centrifuged twice at 15,000 x *g* for 1 min to remove residual solution. The column was placed to a clean 1.5 ml microfuge tube and 50 µl EB Buffer (10 mM Tris-HCl, pH 8.5) was added directly to silica matrix at the center of the column. Plasmid DNA was eluted by centrifugation at 15,000 x *g* for 1 min.

c. Electroporation of *E. coli* cells

Electrocompetent cells (50 µl) stored at -70 °C were thawed on ice before use. Plasmid DNA (~0.02 µg) was mixed gently with the competent cells. The mixture was transferred to a 0.1-cm Gene Pulser® cuvette (Bio-Rad) chilled on ice. The electroporation apparatus (Bio-Rad) was set at 25 µF, 200 ohms, and 1.66 kvolts. The cuvette was dried and placed in the electroporation chamber. Electroporation was

performed by holding down two power buttons until the machine beeped and showed a time constant above 4. Pre-warmed LB medium (1 ml) was added to the cuvette and suspended cells were transferred to a sterile tube and incubated at 37 °C for 15 min. The cells were then spread onto LB plates containing ampicillin (100 μg/ml) and cultured overnight.

d. PCR amplification

DNA fragments were amplified enzymatically using a polymerase chain reaction (PCR). Because of its integral 3'→ 5' proofreading exonuclease activity, the fidelity of Vent® DNA polymerase is 5-15 fold higher than that of *Taq* DNA polymerase. However, Vent® DNA polymerase is poor in its ability to add an additional deoxyadenosine to 3' end of PCR products. Thus, *Taq* DNA polymerase was added at the end of PCR cycles catalyzed by Vent® DNA polymerase to make sure all products had an overhanging deoxyadenosine that is crucial for the subsequent TOPO TA cloning procedure.

PCR reactions were set up in a total volume of 100 μl by mixing 0.2 mM dNTPs, Vent® DNA polymerase (2 units), primer 1 (0.5 μM), primer 2 (0.5 μM), and template DNA (~0.1 μg) in 1 X ThermoPol Reaction Buffer (supplied with Vent® DNA polymerase). The PCR amplification reaction was set to denature DNA templates at 95 °C for 5 min, followed by 30 cycles of reactions (denatured at 95 °C for 0.5 min, annealled at 65 °C for 0.5 min and extended at 72 °C for 0.5 min). After completion of amplification cycles, *Taq* DNA polymerase (2.5 units) was added and samples were incubated at 72 °C for another 20 min. Samples were stored at -20 °C.

e. Recovery of DNA fragment from agarose gels

DNA fragments from PCR amplification or restriction digestion were recovered from agarose gels after the fragments were separated by electrophoresis based on size. DNA binds to silica in high concentrations of chaotropic salt and elutes in low salt solutions. A GENECLEAN II kit based on this mechanism was used. DNA bands from ethidium bromide-stained agarose gel were visualized using long wave UV light and excised with a razor blade. The weight of gel slices was used to determine approximate volume (0.1 g equals 100 µl). Three volumes of 6 mM NaI solution were added followed by incubation at 50 °C for complete dissolution of gel slices. GLASSMILK, an aqueous suspension of proprietary silica matrix used for DNA binding, was added and suspended at 37 °C for 5 min by gentle inversion. GLASSMILK with bound DNA was pelleted with a short centrifugation (30 sec) at 15,000 x *g* and was washed three times with cold NEW Wash solution containing NaCl, Tris, EDTA and ethanol. After the final wash, the silica pellet was dried at 22 °C for 10 min; sterilized distilled H_2O (25 µl) was added and the GLASSMILK was resuspended. GLASSMILK was pelleted by centrifugation at 15,000 x *g* for 30 sec and the supernatant containing eluted DNA was removed and saved.

f. TOPO TA cloning

TOPO TA cloning provides an efficient, rapid, one-step strategy to insert *Taq* polymerase-amplified PCR products into a plasmid vector. Linearized pCR®2.1-TOPO® vector supplied by the manufacturer is covalently bound with topoisomerase I. The tyrosine residue at position 274 of this enzyme forms a phospho-tyrosyl bond with the 3' phosphate of the overhanging deoxythymidine residue on the linearized vector. This bond is attacked by the 5'-hydroxyl group of PCR products to release topoisomerase I. A

phosphodiester bond then forms to ligate the PCR-amplified fragment with the linearized vector. pCR®2.1-TOPO® vector contains an ampicillin resistant gene that allows for selection of cells containing this plasmid. The TA cloning site is located inside the LacZα gene so that the insertion of PCR products disrupts its expression and allows for selection using X-Gal for positive clones. PCR products were separated on agarose gels and recovered using the GENECLEAN II kit as described above. The TOPO cloning reaction was performed in a final volume of 6 µl by mixing PCR product (0.5-4 µl), salt solution (1 µl) and TOPO vector (1 µl) and the mixture was incubated at 22 °C for 5 min. The reaction mixture (2 µl) was added to One Shot® chemically competent *E. coli* cells and mixed gently. After incubation on ice for 30 min, cells were heat-shock treated at 42 °C for 30 sec and immediately transferred to ice. SOC medium (250 µl) was added to the cells and the tube was shaken (200 rpm) at 37 °C for 1 h. A selective plate (LB plates containing 100 µg ampicillin/ml) was prepared by spreading 40 µl X-gal (40 mg/ml) and was warmed at 37 °C for 1 h. Cell culture (10-50 µl) was spread on the selective plate and incubated at 37 °C overnight. Colonies with white or light blue color were picked for further analysis.

g. DNA sequencing and analysis

DNA sequencing was performed to confirm the fidelity of PCR-amplified products. Recombinant pCR2.1-TOPO plasmids prepared using the QIAprep® Miniprep Kit were sent for commercial DNA sequencing at the Dalhousie-NRC Institute for Marine Biosciences Joint Laboratory or at Cortec DNA Service Laboratories, Inc. (Queen's University, Kingston, Ontario). DNA sequences obtained were analyzed using Jellyfish and DNAsist DNA analysis software.

h. Subcloning of DNA fragments

Recombinant pCR2.1-TOPO plasmids were digested at both ends of the inserted PCR fragment using appropriate restriction enzymes. PCR products were recovered from agarose gels as described. A mammalian expression vector, pcDNA3.1/Myc-His(+), was digested with the same set of restriction enzymes and the linearized DNA fragment was separated on agarose gel and recovered. A sticky-end ligation reaction was performed with a mixture of 800 units T4 DNA ligase, linearized vectors and PCR fragments. The reaction occurred at 22 °C for 30 min. The ligation mixture was transferred to a gel column (1% agarose gel in 0.1 M glucose) to remove ions from the DNA sample. The ligation mixture was then electroporated into competent E. coli cells. Colonies grown on a LB plate containing 100 μg ampicillin/ml were picked as positive clones for verification.

6. Construction of expression vectors

Full cDNA sequences of PSS I (GenBank™ accession number A41680) and PSS II (GenBank accession number BAA20355) were kindly provided by Dr. Osama Kuge (Kuge et al., 1991; Kuge et al., 1997; Kuge et al., 1991; Kuge and Nishijima, 1997). *Xho*I and *Apa*I sites were engineered into the 5'- and 3'- ends of the full coding sequence of PSS I by PCR amplification with primer 1, 5' –GACCTCGAGATGGCGTCGTGCGTG-GGGAGCCGG-3' and primer 2, 5' -GCAGGGCCCTTTCTTTCCAACTCCATTGGTG-AC-3' respectively. Similarly, *Eco*RI and *Sac*II sites were added to the 5'- and 3'- ends of the full coding sequence of PSS II by PCR amplification with primer 1, 5' -GACGAATTCATGCGGAGGGCCGAGCGCAGAGTC-3' and primer 2, 5' -GCACCG-CGGTGAGGCGGCTGAGGCCCCCTCCTT-3' respectively. PCR-amplified DNA

fragments were cloned into the TA cloning vector pCR2.1-TOPO and checked by DNA sequencing. PSS I and PSS II were subcloned into the pcDNA3.1/Myc-His(+) A and B expression vector respectively to generate the expression plasmids, pcDNA-PSS I and pcDNA-PSS II.

Murine PLSCR1 cDNA (GenBank™ accession number AF159593) was kindly provided by Dr. Peter Sims (Zhou et al., 1997; Wiedmer et al., 2000; Wiedmer et al., 2000). Murine PLSCR2 homolog cDNA was obtained from ATCC EST clone 1191848, and DNA sequencing indicated that all amino acids were the same as those deposited in GenBank except a highly conserved Arg-Lys transition at 178 position (GenBank™ accession number AF015790) (Zhou et al., 1998). *EcoR*I and *Xho*I sites were engineered into the 5'- and 3'- ends of the full coding sequences of PLSCR1 by PCR amplification with primer 1, 5' -GACGAATTCATGGAAAACCACAGCAAGCAAACT-3', and primer 2, 5' -GCACTCGAGCTGCCATGCTCCTGATCTTTGCTC-3'. *Not*I and *Apa*I sites were added to the 5'- and 3'- ends of the full coding sequences of PLSCR2 by PCR amplification with primer 1, 5' -GACGCGGCCGCATGGAGGCTCCTCGCTCAGGAA-CA-3', and primer 2, 5' -GCAGGGCCCCTCACAGCCTTCAAAAAACATGTA-3'. PCR-amplified DNA fragments were cloned into the TA cloning vector pCR2.1-TOPO and checked by DNA sequencing. Full coding sequences were subcloned into the pcDNA3.1/Myc-His(+) A expression vector to generate the expression plasmids, pcDNA-PLSCR1 and pcDNA-PLSCR2.

7. Generation of stable expressing cell lines

a. Detection of over-expressed proteins

(1) Western blotting

For preparations of Myc-tagged proteins, total protein extracts were prepared by lysing cells in 0.5 ml lysis buffer (1% Triton-X-100, 40 μl/ml 5 X protease inhibitor cocktail in TBS) followed by incubation on ice for 10 min. The cell lysate was centrifuged at 15,000 x *g* for 10 min at 4 °C. Protein in a 200 μl aliquot was precipitated with 1 ml cold acetone. Protein concentration of each sample was determined using a micro BCA protein assay. Samples were resolved by SDS-PAGE (10%) and transferred to PVDF membrane. The membrane was incubated with blocking solution (5% skim milk, 0.2% Tween-20 in PBS) at 22 °C for 1 h and transferred to a blotting solution containing anti-c-Myc mAb (1:2,000, v/v) in blocking solution for 2 h of incubation. The blot was rinsed twice in washing buffer (0.2% Tween-20 in PBS) for 5 min each wash and then incubated with HRP-coupled goat anti-mouse secondary antibody (1:10,000, v/v) for 1 h. The blot was rinsed four times with washing buffer for 10 min each wash. Enhanced chemiluminescence was used to detect relevant proteins according to the manufacturer's protocols.

(2) Immunofluorescence detection

CHO-K1 cells grown on glass cover slips were fixed with formaldehyde (3%, w/v) at 22 °C for 15 min and rinsed with 2 ml ammonium chloride in PBS (5 mM) twice. Cells were permeabilized with 0.05% Triton-X-100 (w/v) in PBS at -20 °C for 10 min and incubated in 2 ml PBS containing 1% BSA (PBS-BSA) at 22 °C for 15 min. Cells were incubated with anti-c-Myc mAb (1:500, v/v) in PBS-BSA at 0 °C overnight and rinsed with PBS-BSA. FITC-conjugated goat anti-mouse secondary antibody (2 μg/ml) was added and incubated at 22 °C for 45 min. In some cases, propidium iodide was added 30 min after incubation with secondary antibody and incubated for another 15 min. Cells

were rinsed twice for 15 min with PBS-BSA. Cover slips 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. FITC 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.

(3) PtdSer synthase assay

CHO-K1 cells were seeded at 3 x 10^5 cells per 60 mm dish in 3 ml growth medium for 3 days (80-90% confluence). After removal of the culture medium, a cell lysate was prepared by scraping cells in 0.5 ml ice-cold suspension buffer (250 mM sucrose, 10 mM HEPES buffer, pH 7.5) and sonication for 30 s on ice. Samples were centrifuged at -4 °C at 600 x *g* for 2 min. The assay mixture contained 5 mM $CaCl_2$, 50 mM HEPES buffer (pH 7.5) and 1 μCi [^{3}H]serine (Kuge et al., 1986a). The supernatant from cell extracts (100 μl) was added to an equal volume of pre-warmed assay mixture and incubated at 37 °C for 20 min. Reactions were terminated by adding 1.5 ml of methanol:H_2O (5:4, v/v) and 2 ml chloroform. Samples were mixed thoroughly and centrifuged at 2,000 rpm for 10 min. Lipid was extracted using modified Folch procedure and total radioactivity was determined using the Beckman counter. Protein concentration was measured using a Micro BCA kit.

b. LipofectAMINE2000 transfection

CHO-K1 cells were seeded at 1 x 10^5 cells per 35 mm dish and cultured in 3 ml growth medium for 3 d (to 80-90% confluence). For one reaction, plasmid DNA (1-3 μg) was mixed in 100 μl DEME; lipofectAMINE2000 reagent (4 μl) was diluted in 100 μl DMEM. The two mixtures were quickly combined and incubated at 22 °C for 30 min. CHO-K1 cells were rinsed with and re-seeded in 800 μl DMEM (supplemented with 300

μM proline). DNA-lipofectAMINE2000 complexes (200 μl) were added and cells were incubated at 22 °C for 5 h. Growth medium containing 10% FBS in proline-supplemented DMEM (1ml) was then added and cells were cultured for another 24-48 h before analyses.

c. Dilution cloning

(1) Selection of stable clones over-expressing PSS I or PSS II

CHO-K1 cells were seeded at 5 x 10^4 cells per 35 mm dish and cultured in 3 ml growth medium for 3 days. Cells were transfected with pcDNA-PSS I, pc-DNA-PSS II constructs or corresponding empty vectors using the LipofectAMINE2000 procedure. One day after transfection, the culture medium was removed and cells were rinsed with sterile PBS and treated with 100 μl trypsin (0.25%); detached cells were suspended in 2 ml medium. Cells (1:2,500, 1:1,000 and 1:500 dilutions) were re-seeded in 8 ml growth medium containing G418 sulfate (500-800 μg/ml) in 100 mm dishes. Selection medium was refreshed every 48 h. Cells started to die 1 week after the addition of G418 and 10-14 d after initiation of selection, single isolated clones on the dishes (1:2,500 dilution) were marked under a microscope. Cells from each marked clone were trypsinized and transferred into individual wells of 6- or 12-well culture plates. Selection medium (1 ml) was added to each well and refreshed every 48 h. When cells reached confluence (in about 1 week), each single clone was split. Some cells were used for analysis of protein expression and the remainder were cultured for future use.

Immunofluorescence detection of c-Myc-PSS I and -PSS II was used to identify positive clones. Cells from 16 different clones were seeded in a 16-well chamber slide; each well contained 500 μl selection medium. When clones reached 60-70% confluence,

an immunofluorescence procedure was performed to stain cells over-expressing Myc-tagged proteins. The chambers were then detached from the slides and glass cover slips were used to cover the cells on the slide. Fluorescence was then visualized using an Olympus inverted microscope model IMT2. Clones identified as positive were cultured into 100 mm dishes. When all reached confluence, they were saved in storage vials in liquid nitrogen. Cells over-expressing PSS I and PSS II were maintained in growth medium containing 350 μg G418/ml.

(2) Selection of stable clones over-expressing PLSCR1 or PLSCR2

CHO-K1 cells were seeded at 1 x 10^5 per 35 mm dish and cultured in 3 ml growth medium for 3 d. Expression constructs, pcDNA-PLSCR1 and pc-DNA-PLSCR2, and pcDNA3.1/Myc-His(+) A vector were used to co-transfect CHO-K1 cells with pTK-Hyg vector using the LipofectAMINE2000 procedure. The co-integration of pTK-Hyg vector along with the expression vector into the host genome enables the use of hygromycin (a potent antibiotic) and G418 for more efficient selection of stable cell lines. One day after transfection, the culture medium was removed and cells were rinsed with sterile PBS and detached using 100 μl trypsin (0.25%). Cells were suspended in 2 ml medium and were split into 3-5 dishes (100 mm) containing 8 ml growth medium supplemented with 350 μg G418/ml and 200 μg hygromycin/ml. Selection medium was refreshed every 48 h. Cells started to die 4 days after the addition of antibiotics and 10-14 days after initiation of selection, individual clones were transferred to 6- or 12-well culture plates. Selection medium (1 ml) was added to each well and refreshed every 48 h. Immunofluorescence detection of c-Myc-PLSCR1 and c-Myc-PLSCR2 was performed to identify positive clones as described. Positive clones were cultured into 100 mm dishes and when

confluence was reached, cells were harvested and stored in liquid nitrogen. Cells over-expressing PLSCR1 and PLSCR2 were maintained in regular growth medium containing 350 μg G418/ml and 200 μg hygromycin/ml.

IV. Results

A. Metabolism of serine-derived phospholipids in apoptotic U937 cells

1. Apoptosis and PtdSer externalization in U937 cells induced by camptothecin

Human leukemia U937 cells were induced to undergo apoptosis by treatment with a topoisomerase I inhibitor, camptothecin (CAM) (Rothenberg, 1997; Johnson et al., 1997). Exposure of PtdSer on the surface of the apoptotic cells was detected using annexin-V, a highly specific PtdSer binding protein (Tait and Gibson, 1992; van Engeland et al., 1998). Four hours after induction with 10 μM CAM, significant annexin-V-FITC binding to cells, indicated by green membrane rings (Fig. 8A), was accompanied by reduction of cell size and formation of microvesicles (Fig. 8B) that also bound annexin-V. Membrane integrity was maintained as few annexin-positive cells were labeled with propidium iodide (PI), a membrane-impermeant nuclear dye that enters cells when membrane integrity is lost and stains the nucleus red (Nicoletti et al., 1991).

HL-60 cells in S-phase are more sensitive to induction of apoptosis with CAM (Gorczyca et al., 1993). To increase sensitivity to CAM, we synchronized the U937 cells with 2 mM thymidine to block them at S-phase (Galavazi and Bootsma, 1966). Both unsynchronized and synchronized cells showed time-dependent increases in PtdSer exposure after induction by CAM (Fig. 8C), but compared to unsynchronized cells, synchronized U937 cells showed a higher percentage and earlier exposure of PtdSer. In synchronized cells, PtdSer externalization was detected in 20% of the cells at 2 h after induction while similar PtdSer exposure required 3 h in unsynchronized cells. By 5 hours after induction, about 70% of cells were positive for annexin-V binding in synchronized cells whereas only 35% bound annexin-V in unsynchronized cells. Synchronized and

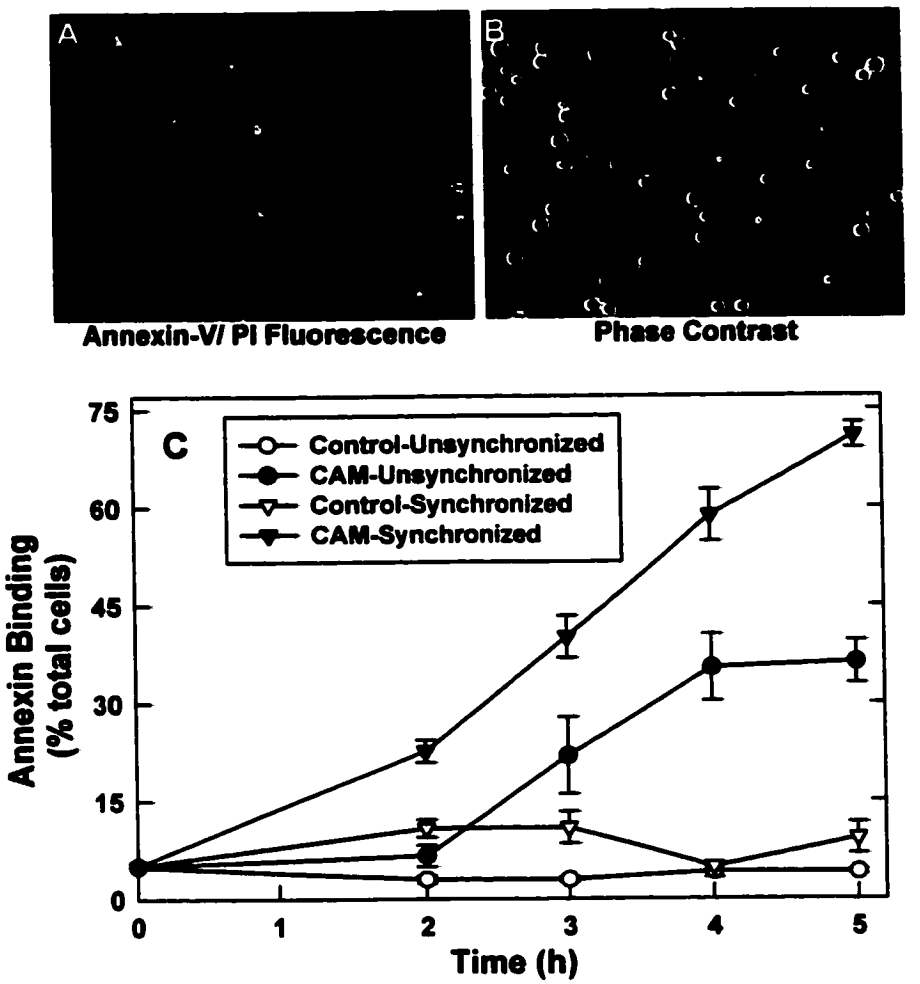

Figure 8. Induction of PtdSer externalization by camptothecin in U937 cells. Thymidine-synchronized cells were treated with 10 μM camptothecin (CAM) for 4 h. PtdSer exposure was detected with annexin-V-FITC/PI staining; both fluorescence (panel A) and phase contrast images (panel B) were recorded at 300X magnification. For panel C, both unsynchronized and synchronized cells were treated without or with 10 μM CAM for various periods of time. Approximately 500 cells were assessed for annexin-V binding in each sample. Values are mean ± SEM for 3 separate experiments.

unsynchronized U937 cells showed similar basal levels of apoptosis without CAM treatment. Based on these observations, we used synchronized cells to induce apoptosis by CAM in all subsequent experiments. Under these conditions, caspase-3, caspase-1 and PARP all were cleaved proportional to CAM concentration (Fig. 9A) and time of exposure (Fig. 9B). As shown, pro-caspase-3, an inactive 32 kDa protein, was cleaved to active 17 kDa and 12 kDa fragments during CAM-induced apoptosis; PARP, a direct substrate of caspase-3, was cleaved to its inactive 85 kDa form indicating activation of caspase-3 activity; together these are hallmarks of apoptosis (Tewari et al., 1995). Our data also showed that caspase-1 was cleaved to its active 20 kDa form during CAM-mediated apoptosis in U937 cells.

2. Stimulation of PtdSer synthesis in apoptotic U937 cells

To assess PtdSer biosynthesis in U937 cells during CAM-induced apoptosis, cells were pulse labeled for 4 h with [^{3}H]serine along with various concentrations of CAM. Total PtdSer biosynthesis was stimulated in apoptotic cells with CAM concentrations up to 25 μM (Fig. 10A). PtdSer isolated from cells increased with time in both control and CAM-induced apoptotic cells with the major increase in the first 3 h of CAM treatment (Fig. 10B). Interestingly, we noticed radiolabel was recovered not only in cells but also in the cell-free medium collected after centrifugation. This suggested formation of small vesicles from cells that were too small to be pelleted by the relatively mild centrifugation. Annexin-V-FITC binding assays confirmed the existence of vesicles with externalized PtdSer in the cell-free medium. Newly radiolabeled PtdSer accumulated in the medium vesicles with increasing concentrations of CAM (Fig. 10A), while cellular PtdSer radiolabeling decreased with 0-15 μM CAM treatment and then increased slightly

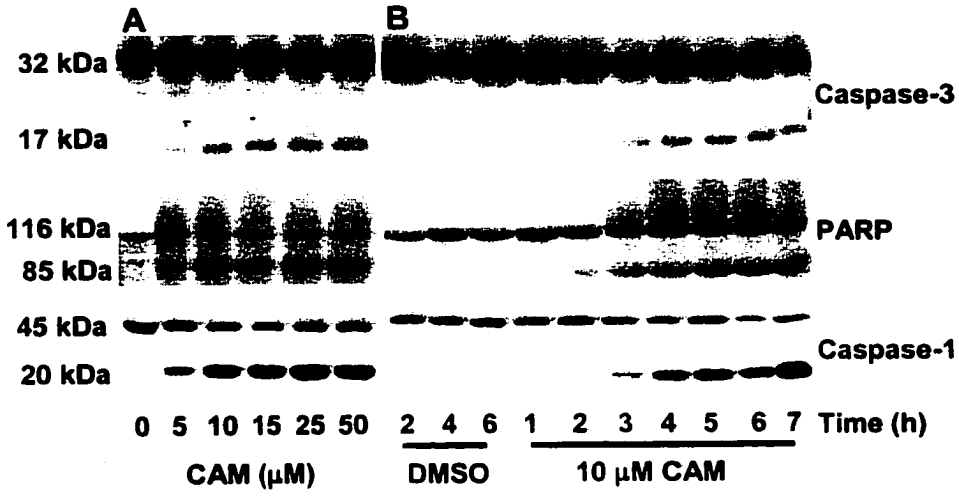

Figure 9. Cleavage of caspase-3, PARP and caspase-1 in camptothecin-treated U937 cells. In panel A, cells were treated with various concentrations of CAM for 4 h. In panel B, cells were treated without or with 10 μM CAM for various periods of time. Proteins were extracted, subjected to SDS-PAGE and transferred to PVDF membranes. Protein bands were detected with antibodies specific for human caspase-3 (top gel with bands at approximately 32 and 17 kDa), PARP (middle gel with bands at approximately 116 and 85 kDa) and caspase-1 (bottom gel with bands at approximately 45 and 20 kDa).

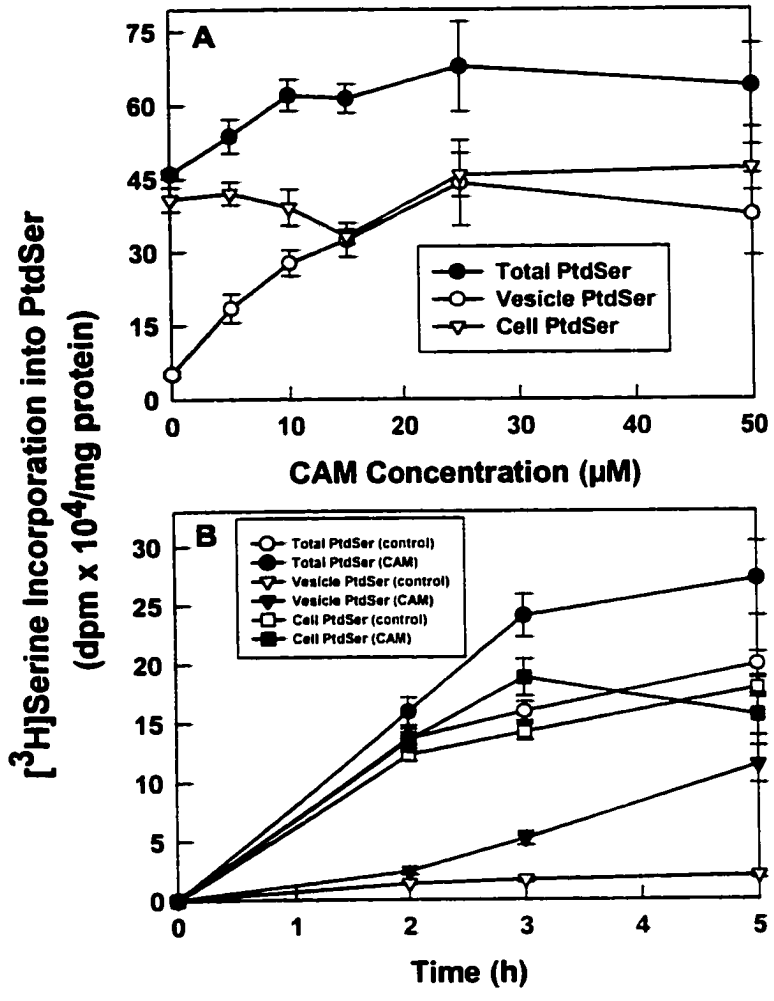

Figure 10. Stimulation of PtdSer synthesis and accumulation in apoptotic vesicles following treatment of U937 cells with camptothecin. In panel A, cells were incubated for 4 h with various concentrations of CAM and 12 μCi [^{3}H]serine. In panel B, cells were incubated for various times with or without 10 μM CAM and 6 μCi [^{3}H]serine. Cells and apoptotic vesicles were separated; lipids were extracted, separated by TLC. Total [^{3}H]serine incorporation into PtdSer and total protein were calculated as the sum from the cell pellet and medium vesicles. Values are mean ± SEM for n=6.

when higher concentrations were used. There was also a significant time-dependent accumulation of radiolabeled PtdSer in medium after treatment with 10 μM CAM (Fig. 10B). Treatment with CAM for 5 h resulted in an 8-fold increase of newly radiolabeled PtdSer in the medium.

We also examined the synthesis of other phospholipids during CAM-induced apoptosis. When U937 cells were treated with 10 μM CAM for 4 h, little change was observed in total SM synthesis from [^{3}H]serine or *de novo* synthesis of PtdEtn from [^{14}C]ethanolamine; PtdCho synthesis decreased about 20% (Fig. 11A). Little change was observed in synthesis of PtdEtn formed through decarboxylation of PtdSer labeled with [^{3}H]serine (data not shown). Interestingly, the accumulation of all four phospholipids in the medium containing apoptotic bodies increased substantially compared to untreated controls (Fig. 11B); however, newly synthesized PtdSer preferentially appeared in apoptotic bodies with a 6-fold increase compared to 3-fold for SM, 2-fold for PtdCho and 1.8-fold for PtdEtn. Thus, our data indicated that during CAM-induced apoptosis in U937 cells, PtdSer synthesis was stimulated and newly synthesized PtdSer was transferred preferentially to the cell surface and into apoptotic vesicles budding from cells.

3. Blockage of stimulation of PtdSer synthesis and translocation by caspase inhibitor

Caspases are key "executioners" of apoptosis and exist in resting cells as inactive proenzymes. Activation of caspases during apoptosis involves proteolytic formation of two fragments from the proenzymes and the active enzyme is a heterotetramer containing two small and two large subunits (Cohen, 1997). Effective inhibitors that mimic natural substrate sequences of caspases have been developed (Villa et al., 1997). z-VAD-fmk, a

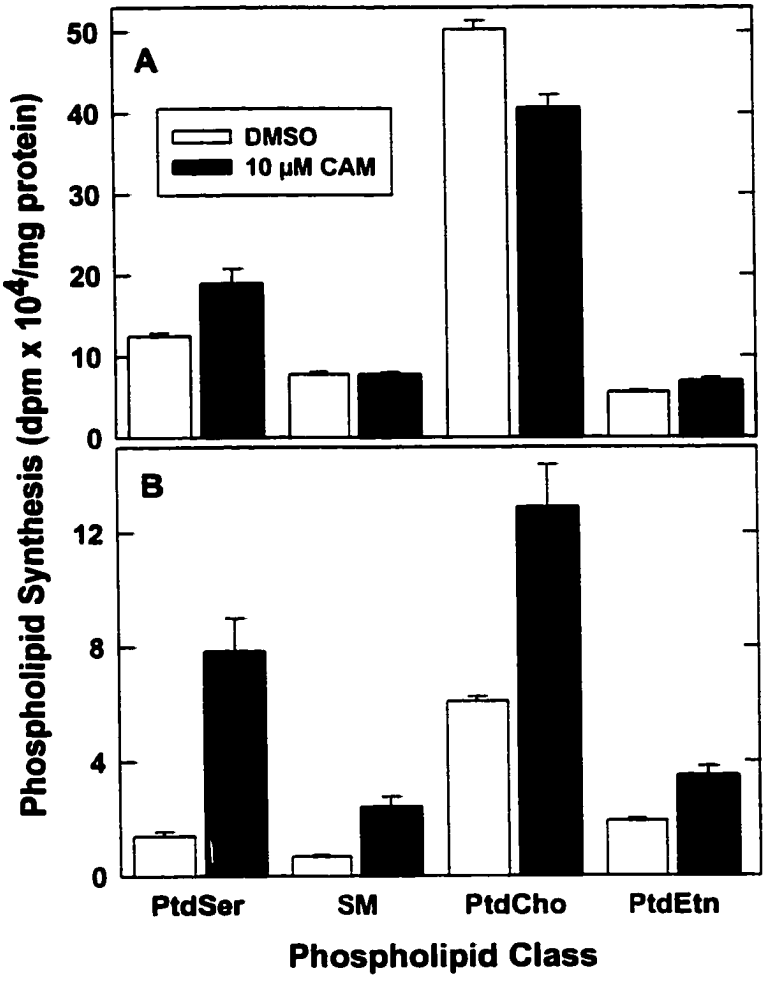

Figure 11. Synthesis of phospholipids and accumulation in apoptotic vesicles following treatment of U937 cells with camptothecin. Thymidine-synchronized U937 cells were pulse labeled for 4 h with 6 μCi [^{3}H]serine, 6 μCi [^{3}H]choline or 6 μCi [^{14}C]ethanolamine in the presence of 10 μM CAM. Cells and apoptotic vesicles were separated. Lipids were extracted and separated by TLC. Panel A shows total labeling of phospholipids and panel B is labeled phospholipids in apoptotic vesicles. In both panels, sphingomyelin (SM) is based on labeling from [^{3}H]serine and PtdEtn shown was derived from [^{14}C]ethanolamine. Values are mean ± SEM for n=6.

broad-range inhibitor of caspases, is effective on most members of the caspase family (Garcia-Calvo et al., 1998). As shown in Figure 12A, we found that 100 μM z-VAD-fmk completely inhibited CAM-induced condensation and fragmentation of the cell nucleus. When cells were incubated with different concentrations of z-VAD-fmk along with 10 μM CAM, there was a shift in fragmentation of caspase-3 from a smaller 17 kDa component to one of slightly larger molecular weight indicating cleavage at alternative sites (Fig. 12B, top panel). Appearance of the smaller cleavage fragments of PARP and caspase-1 also were blocked with increasing concentrations of inhibitor and nearly complete reversal of the fragmentation was observed with 100 μM z-VAD-fmk (Fig. 12B, middle and bottom panels). Less PARP cleavage at higher z-VAD-fmk concentrations indicated that caspase-3 activity was blocked because PARP is believed to be a direct substrate of caspase-3 (Tewari et al., 1995).

CAM-induced PtdSer exposure determined as annexin-V binding also was inhibited by z-VAD-fmk in a concentration dependent manner (Fig. 13). Membrane budding was similarly inhibited by increasing concentrations of z-VAD-fmk. When U937 cells were incubated with 10 μM CAM and various concentrations of z-VAD-fmk, stimulation of total PtdSer biosynthesis was inhibited proportional to the concentration of the inhibitor and accumulation of radiolabeled PtdSer in apoptotic bodies also was blocked (Fig. 14A). When U937 cells were incubated with 50 μM z-VAD-fmk and 10 or 25 μM CAM, the accumulation of radiolabeled PtdSer in medium was reduced to control levels (Fig, 14B). Thus, stimulation of PtdSer biosynthesis and externalization by CAM seems to be a specific event in apoptosis that requires the activation of caspases.

Figure 12. Prevention of camptothecin-induced apoptosis by caspase inhibitor, z-VAD-fmk, assessed by changes in nuclear morphology and cleavage of caspase-3, PARP and caspase-1. In panel A, U937 cells were treated with 10 μM CAM in combination with 100 μM z-VAD-fmk for 4 h. Nuclei were stained with Hoechst 33258 as described in Methods. For panel B, cells were treated for 4 h with DMSO or 10 μM CAM in combination with various concentrations of z-VAD-fmk. Proteins were extracted, subjected to SDS-PAGE and transferred to PVDF membrane as described in Methods. Protein bands were detected with antibodies specific for caspase-3 (top panel), PARP (middle panel) and caspase-1 (bottom panel).

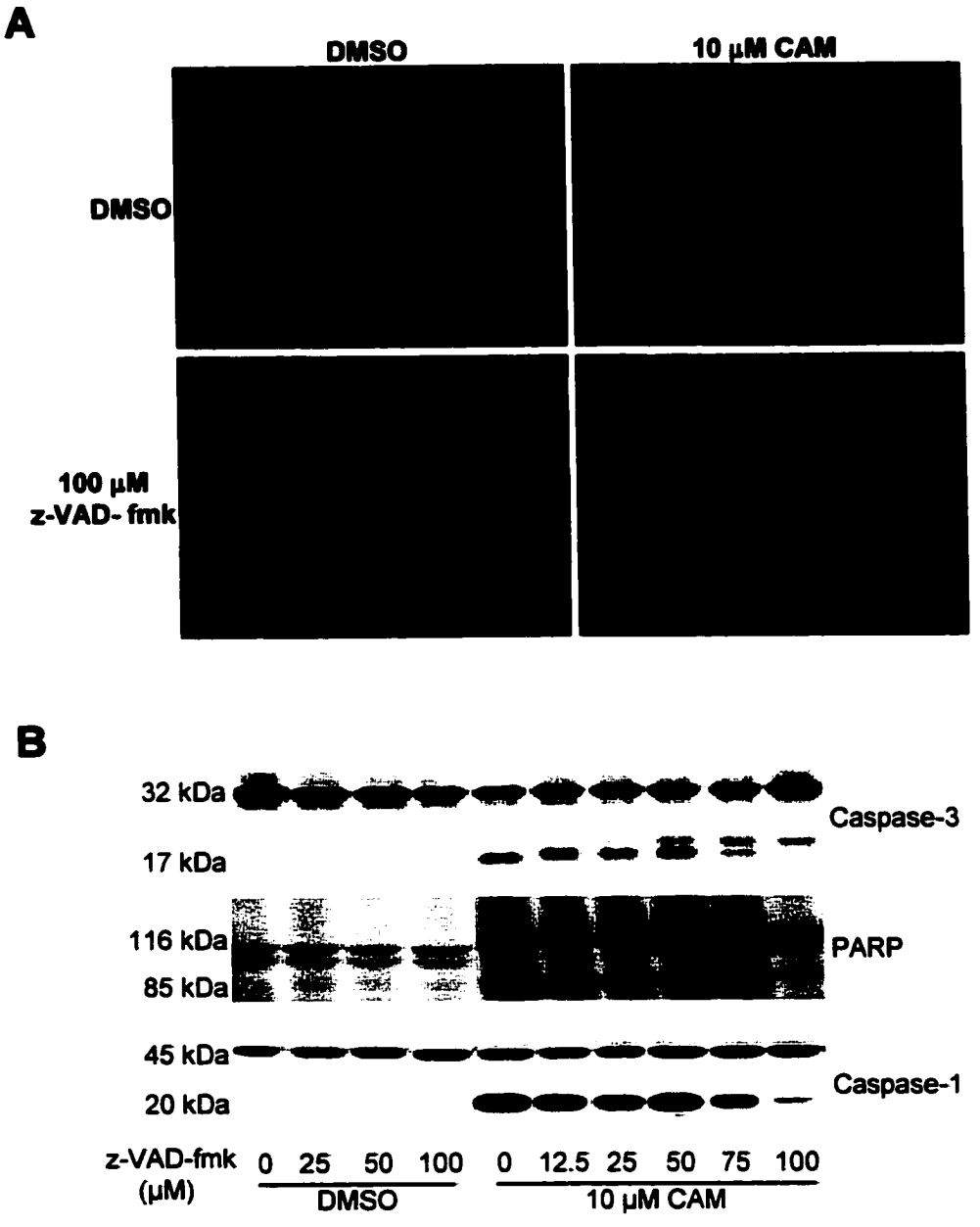
A
DMSO
10 μM CAM
DMSO
100 μM
z-VAD- fmk
B
32 kDa
Caspase-3
17 kDa
116 kDa
PARP
85 kDa
45 kDa
Caspase-1
20 kDa
z-VAD-fmk
(μM)
0 25 50 100
0 12.5 25 50 75 100
DMSO
10 μM CAM

Figure 12

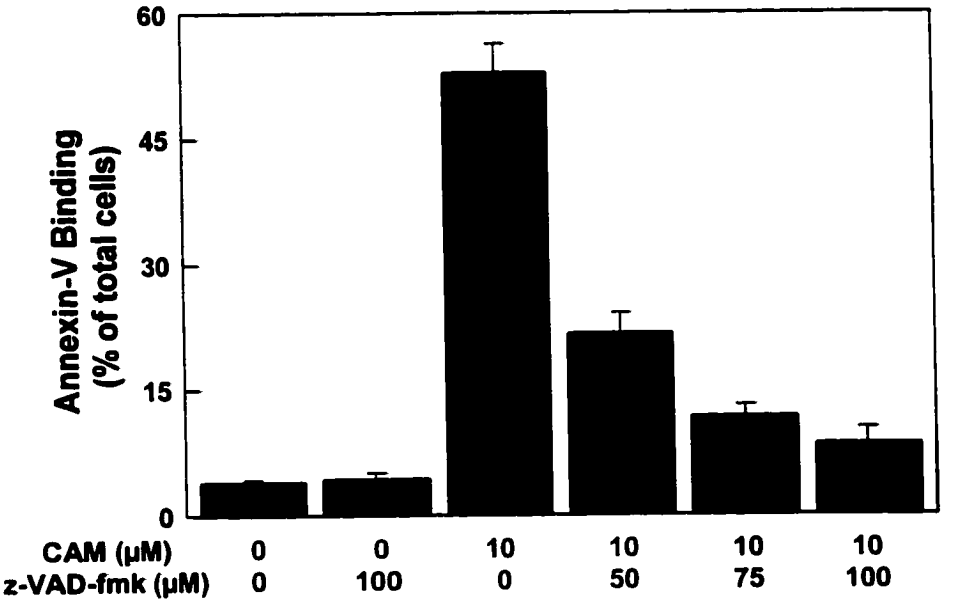

Figure 13. Prevention of camptothecin-induced PtdSer externalization in U937 cells by z-VAD-fmk. U937 cells were treated without or with 10 μM CAM in combination with various concentrations of z-VAD-fmk for 4 h. Cell surface exposure of PtdSer was assayed with annexin-V-FITC and PI staining, with approximately 500 cells counted in each sample. Values are mean ± SEM for 3 separate experiments.

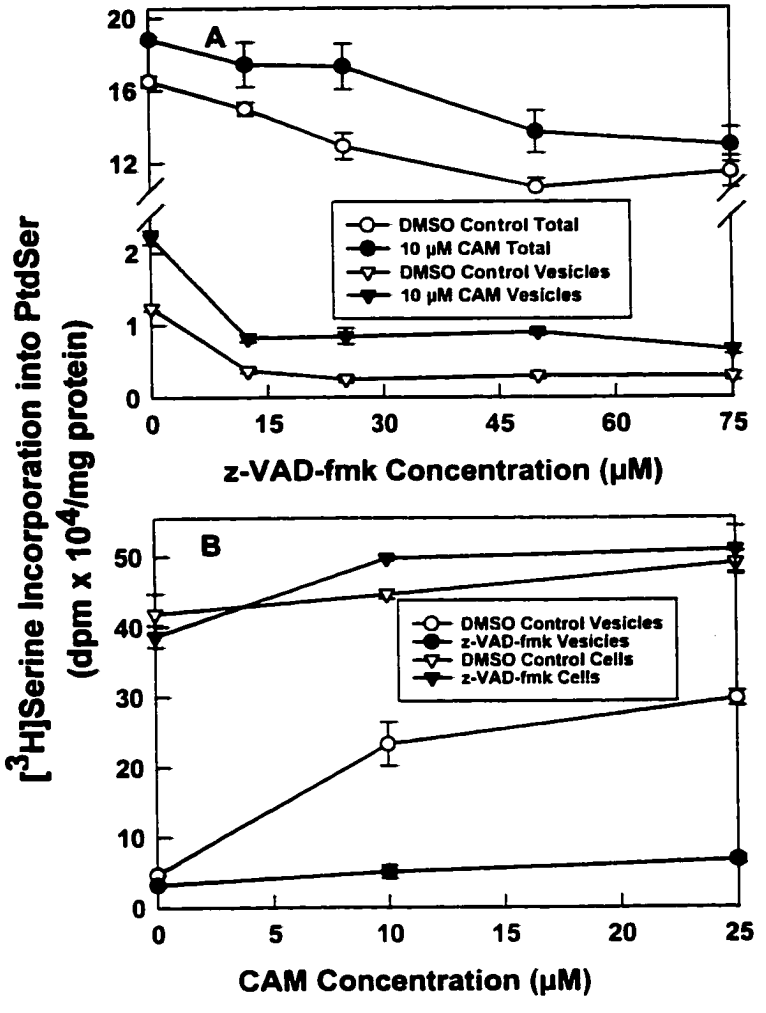

Figure 14. Prevention of camptothecin-stimulated PtdSer synthesis by z-VAD-fmk. In panel A, U937 cells were preincubated for 1 h with various concentrations of z-VAD-fmk followed by 4 h incubation with 6 μCi [^{3}H]serine and 10 μM CAM or DMSO (control). In panel B, CAM at various concentrations was added simultaneously with or without 50 μM z-VAD-fmk and 12 μCi [^{3}H]serine. After incubation for 4 h, cells and apoptotic vesicles were obtained as described in Methods and lipids were extracted and separated by TLC. Values are mean ± SEM for n=6.

4. Stimulation of PtdSer synthesis and movement by other apoptotic stimuli

To provide additional evidence for apoptosis-specific stimulation of PtdSer synthesis during programmed cell death, we induced apoptosis in U937 cells with TNF-α and cycloheximide (CHX) or exposure to UV irradiation at 254 nm for 5 min (Bratton et al., 1997; Vanags et al., 1996). Morphological changes such as PtdSer externalization and membrane budding (Fig. 15, A-F) and nuclear changes were observed (Fig. 16, panels A, C and E) indicating induction of cell death. Four hours after induction, >95% of cells showed apoptotic characteristics. The nuclear changes in UV-irradiated cells were effectively blocked by z-VAD-fmk (Fig. 16D), whereas the addition of z-VAD-fmk led to massive loss of TNF-α–treated cells due to cell fragmentation (Fig. 16F). This observation is consistent with the previous suggestion that although z-VAD-fmk can inhibit caspase-mediated apoptosis induced by TNF-α/CHX in U937 cells, the combination leads to rapid necrosis mediated by excessive formation of reactive oxidative species (Khwaja and Tatton, 1999).

When apoptosis was induced by UV irradiation, a 50-fold increase in radiolabeled PtdSer was observed in microvesicles and overall labeling of PtdSer was 3-fold higher than in control cells. TNF-α alone can induce apoptosis less efficiently due to competition between cell survival and death pathways activated simultaneously by TNF-α pathways (Ashkenazi and Dixit, 1998). With addition of CHX to block the cell survival pathway, apoptosis was more efficiently induced (Kull, Jr. and Besterman, 1990). CHX alone had little effect on total PtdSer biosynthesis and TNF-α treatment alone induced a 1.5-fold increase in total PtdSer synthesis and 10-fold increase in PtdSer accumulation in vesicles (Fig 17, upper panel). When cells were incubated simultaneously with TNF-α and CHX, significant accumulation of radiolabeled PtdSer in apoptotic bodies was observed in treated cells with a nearly 36-fold increase at 4 h. Total PtdSer synthesis rate

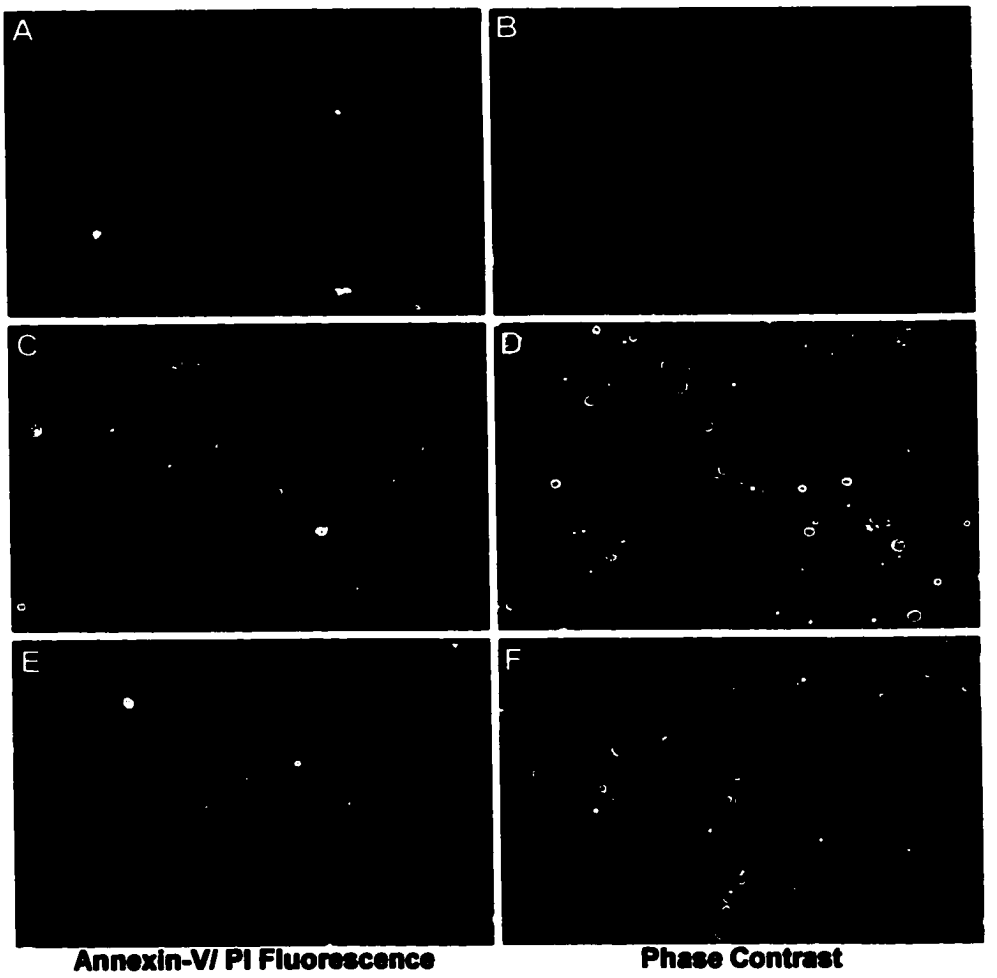

Figure 15. PtdSer externalization following UV irradiation or TNF-α /cycloheximide-induced apoptosis. Apoptosis was induced in U937 cells by exposure to UV light at 254 nm for 5 min or to 25 ng TNF-α with 1 μg cycloheximide (CHX)/ml. After 4 h incubation, PtdSer exposure and membrane integrity were detected using annexin-V-FITC/PI staining as described in Methods. Both fluorescence (panels A, C and E) and phase contrast (panels B, D and F) images were obtained for the same field at 300X magnification. Panels A and B, controls with no exposure; panels C and D, UV irradiation; panels E and F, TNF-α and CHX administration.

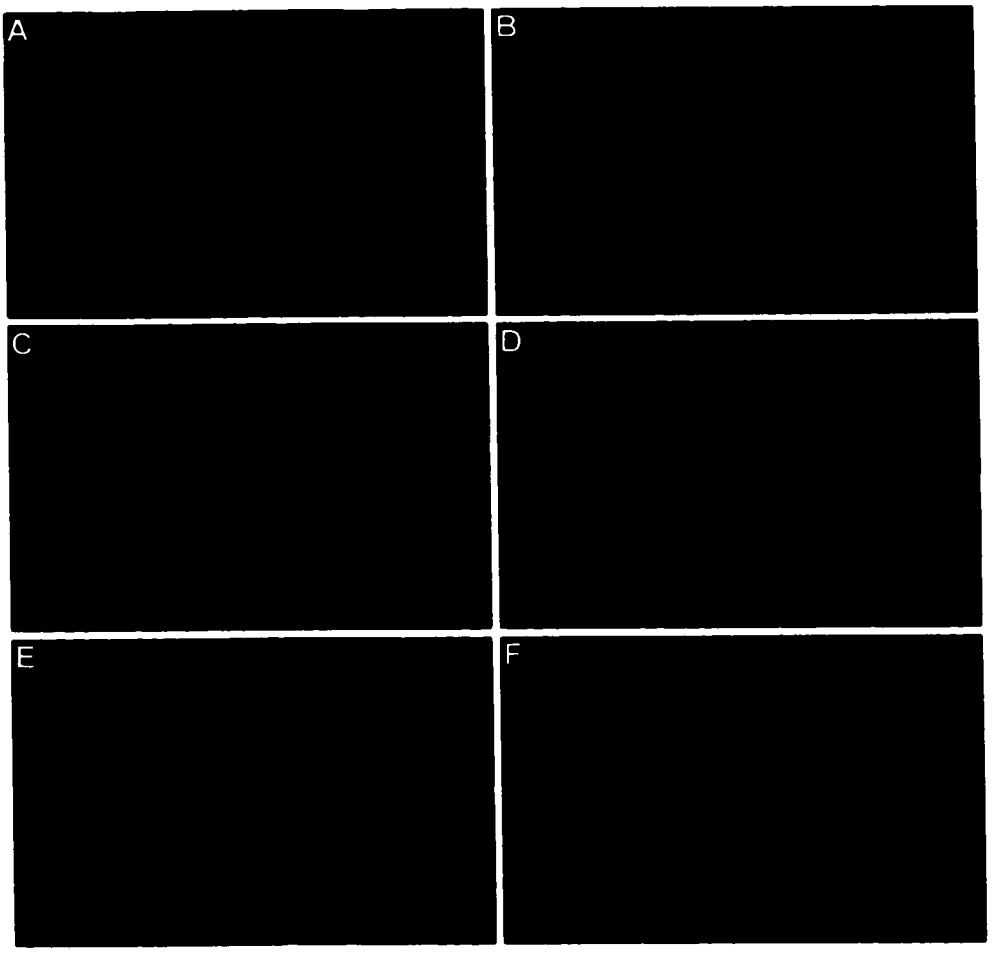

Figure 16. Blockage of nuclear changes induced following UV irradiation or TNF-α/cycloheximide treatment by z-VAD-fmk. Apoptosis of U937 cells was induced by exposure to UV light at 254 nm for 5 min or to 25 ng TNF-α with 1 μg CHX/ml. Cells were incubated with (panels B, D and F) or without (panels A, C and E) the presence of 100 μM z-VAD-fmk. After 4 h of incubation, nuclear condensation and fragmentation were detected using Hoechst 33258 staining at 300X magnification. Panels A and B, no treatment; panels C and D, UV irradiation; panels E and F, TNF-α and CHX administration.

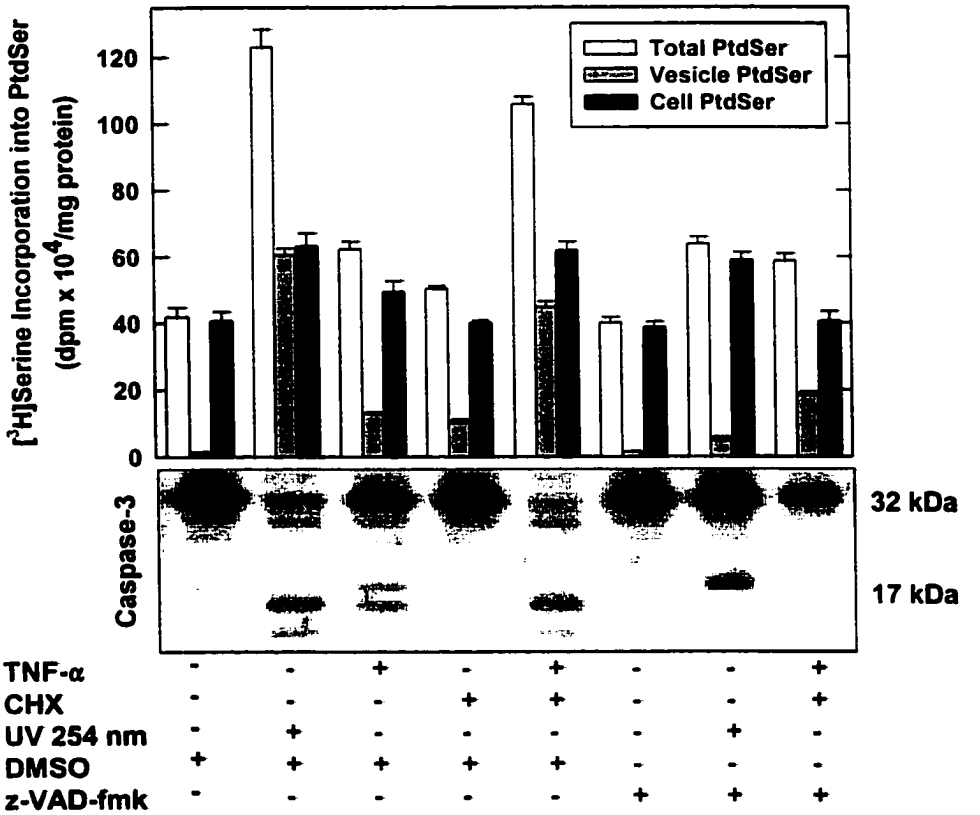

Figure 17. Stimulation of PtdSer biosynthesis and accumulation in apoptotic vesicles following UV irradiation or TNF-α/cycloheximide-induced apoptosis. Apoptosis was induced by exposure to UV light at 254 nm for 5 min or to TNF-α (25 ng/ml) with or without CHX (1 μg/ml); 50 μM z-VAD-fmk was added to some samples as indicated. In the upper panel, cells were labeled for 4 h with 12 μCi [^{3}H]serine and cells and vesicles were collected as described in Methods. Lipids were extracted and separated by TLC and radioactivity in PtdSer was determined. Values are mean ± SEM for n=6. In the lower panel, cells were incubated as indicated for 4 h, proteins were extracted, subjected to SDS-PAGE and transferred to PVDF membrane. Protein bands were detected with antibodies specific for human caspase-3.

was approximately 2.5-fold higher in TNF-α/CHX induced apoptotic cells than in control cells. Although z-VAD-fmk alone showed no effects on total PtdSer synthesis or accumulation in vesicles compared to controls, blockage of apoptosis with 50 μM z-VAD-fmk abrogated accumulation of labeled PtdSer in medium from UV treated cells and partially inhibited appearance of PtdSer in apoptotic vesicles in TNF-α/CHX treated cells. Total PtdSer biosynthesis also was reduced greatly in both cases.

Ceramide formation has been implicated in apoptosis mediated by UV irradiation or TNF-α pathways (Wright et al., 1996); we observed a decrease in SM labeling from [^{3}H]serine in apoptotic cells induced by UV irradiation (40% decrease) or TNF-α/CHX (66% decrease) compared to controls (Fig. 18A). This was reversed in UV treated cells treated with z-VAD-fmk but remained decreased in TNF-α/CHX treated cells despite the presence of the caspase inhibitor. [^{3}H]serine-derived SM also accumulated in apoptotic bodies with a 12-fold increase with UV irradiation and 3-fold with TNF-α/CHX treatment; z-VAD-fmk partially reversed SM accumulation in apoptotic bodies in UV treated cells but had no effect on TNF-α/CHX treated cells. PtdEtn formation through PtdSer decarboxylation showed little change in apoptotic cells induced by UV irradiation or TNF-α/CHX similar to the CAM-induced apoptosis, but [^{3}H] serine-derived PtdEtn accumulation in apoptotic bodies increased 26-fold with UV irradiation and 17-fold with TNF-α/CHX treatment (Fig. 18B). z-VAD-fmk completely reversed movement of radiolabeled PtdEtn to vesicles in UV treated cells but had little effect on its movement in TNF-α/CHX treated cells.

Phospholipid composition of U937 cells was determined following induction of apoptosis (Table 1). In control U937 cell, the major phospholipids were PtdCho (37%) and PtdEtn (16%). PtdSer was about 6% of total phospholipid whereas SM was 8%.

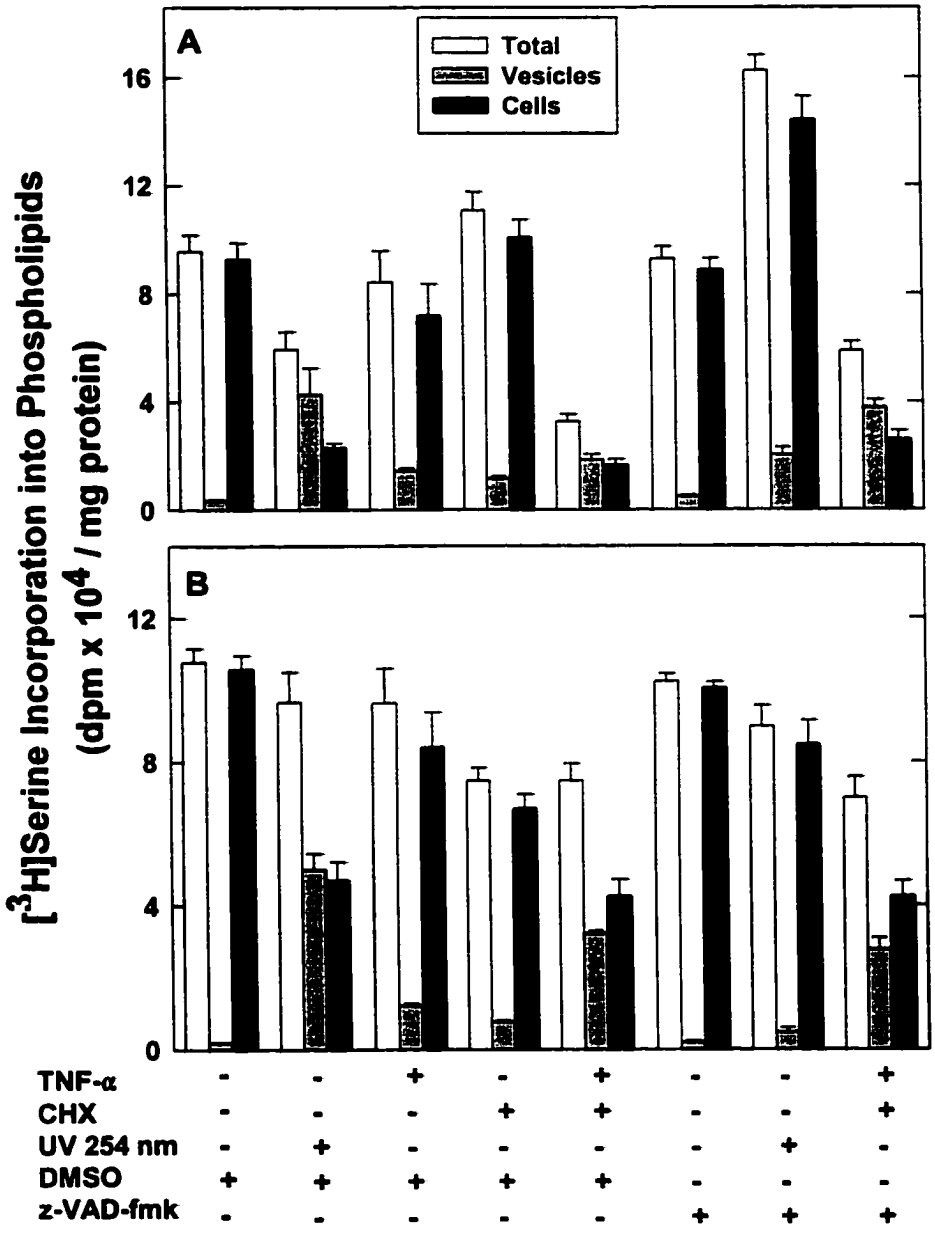

Figure 18. Effects of UV irradiation or TNF-α/cycloheximide-induced apoptosis on serine incorporation into SM and PtdEtn. Incubation and isolation conditions were as described for Figure 17. Radioactivity in SM (panel A) and PtdEtn (panel B) was determined. Values are mean ± SEM for n=6.

Table 1. Phospholipid compositions of U937 cells induced to undergo apoptosis by UV or TNF-α/cycloheximide treatment

Treatment	Percentage of total phospholipids			
	PtdCho	PtdEtn	PtdSer	SM
Control	36.6 ± 0.5	16.4 ± 1.0	5.9 ± 0.5	7.7 ± 0.8
TNF-α/CHX	34.2 ± 1.3*	16.5 ± 1.8	8.9 ± 1.4*	7.9 ± 0.5
UV	35.0± 3.3	15.0 ± 2.3	9.1 ± 1.3*	8.6 ± 0.7

* $p < 0.005$ versus control

Apoptosis was induced in U937 cells by exposure to UV light at 254 nm for 5 min or to 25 ng TNF-α with 1 μg CHX/ml. Cells were then cultured for 4 h. Cells and apoptotic vesicles were collected and lipids were extracted. Individual phospholipid from total lipid extract (cellular and vesicular fractions) was isolated by TLC and the mass was determined chemically by phosphorus analysis as described in Methods. Data are percentage of the mass of each phospholipid relative to that of total lipid extracts. Values are mean ± SEM for n=5. The level of significance was assessed with a Student's *t*-test for unpaired data.

When cells were induced to undergo apoptosis following treatment with UV irradiation or TNF-α/CHX, the mass of PtdSer was increased to 9% (1.5-fold) whereas no change or a slight decrease was observed in the content of PtdCho and PtdEtn. SM mass was unchanged in TNF-α/CHX-treated cells and in UV-treated cells. Thus, PtdSer mass was specifically increased in U937 cells in response to UV or TNF-α/CHX induced apoptosis.

B. PtdSer biosynthesis in CHO-K1 cells during UV-induced apoptosis

CHO-K1 cells are susceptible to UV-induced apoptosis (Tzang et al., 1999a; Tzang et al., 1999b). Following exposure to UV light, CHO-K1 cells developed apoptotic morphology and biochemical changes such as cleavage of PARP within 8-12 h (Fig. 19A). Synthesis of serine-derived phospholipids was monitored during the process of UV-induced apoptosis using [^{3}H]serine. A time-dependent increase in PtdSer formation was observed in cells exposed to UV irradiation (2-fold increase by 16 h) whereas the level of PtdEtn derived from decarboxylation of PtdSer decreased slightly (Fig. 19B). As UV-irradiation did not increase serine uptake (data not shown), the stimulation of PtdSer formation likely resulted from direct regulation of the serine base-exchange reaction at the ER or MAM through the activities of PtdSer synthases; a decrease in PtdSer decarboxylation or an increase in intracellular levels of serine was not responsible. Incorporation of serine into SM was also stimulated in a time-dependent manner following UV irradiation (Fig. 19B). Membrane blebbing into microvesicles is a typical morphological feature of apoptotic cells at late stages of cell death (Mills et al., 1999; Hacker, 2000). When cells were separated from cell-free medium containing microvesicles at 24 h after UV irradiation, PtdSer and SM recovered from vesicles were

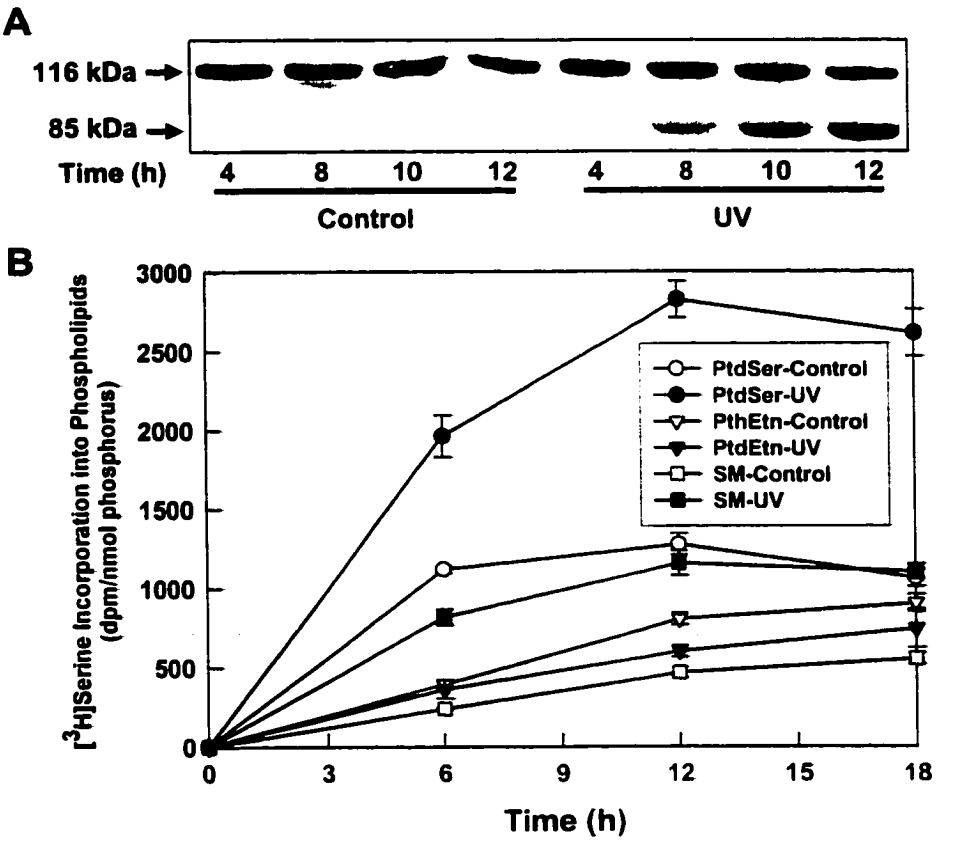

Figure 19. Induction of apoptosis in CHO-K1 cells by UV irradiation and metabolism of serine-derived phospholipids. For panel A, CHO-K1 cells were treated with or without UV light and cultured for the times indicated. Proteins were extracted and separated (20 μg) using SDS-PAGE. Immunoblotting was performed with anti-human PARP pAb as described in Methods. In panel B, CHO-K1 cells were grown in serine-free DMEM. Following treating cells with or without UV light, cells were incubated with 12 μCi [^{3}H]serine for the periods of time indicated and harvested as described in Methods. Lipids were extracted and separated by TLC and radioactivity in PtdSer, PtdEtn and SM was normalized relative to total phosphorus. Data are mean ± SEM of 6 samples.

6-fold higher than control levels without UV treatment (Table 2), whereas a 1.5-fold increase was observed in the levels of serine-derived PtdEtn in vesicles. Thus, in contrast to U937 cell, biosynthesis of SM is also stimulated along with PtdSer in CHO-K1 cells following UV-induced apoptosis and both phospholipids were transported to apoptotic vesicles at high levels.

Blockage of PARP cleavage by a caspase inhibitor, z-VAD-fmk, indicated that UV-induced apoptosis was inhibited (Fig. 20A). However, stimulation of PtdSer biosynthesis was not reversed by the inhibition of progression of apoptosis by z-VAD-fmk (Fig. 20B), indicating activation of caspases is not required for the up-regulation in PtdSer formation. This observation apparently contradicts the caspase-dependent PtdSer stimulation we observed using U937 cells. A slight increase in SM biosynthesis was observed with the presence of z-VAD-fmk (Fig. 20C).

C. Involvement of PtdSer synthases in PtdSer biosynthesis during apoptosis

1. Stable expression of PtdSer synthase I or II in CHO-K1 cells

To study the involvement of PtdSer synthase (PSS) activity in regulating PtdSer formation during UV-induced apoptosis, we established stable cells over-expressing c-Myc-tagged PSS I and PSS II. Transient expression of PSS I gave protein bands at 42 kDa and 80 kDa (Fig. 21A) consistent with PSS I being a highly hydrophobic protein that migrates faster in SDS-PAGE than predicted by the theoretical mass of 55 kDa (Saito et al., 1996). Transient expression of PSS II gave a protein band close to its calculated size (55 kDa) with extensive polymerization resulting in smeared bands migrating at high MW (Stone and Vance, 2000). *In vitro* serine base-exchange activities were 2-3 fold higher in CHO-K1 cells stably over-expressing PSS I or PSS II than in control cells transfected with vector alone (Fig. 21C).

Table 2. Effects of UV-induced apoptosis on distribution of serine-derived phospholipids in cellular and vesicular fractions of CHO-K1 cells

	[^{3}H]Serine incorporation into phospholipids (1×10^3 dpm/nmol phosphorus)		
	PtdSer	PtdEtn	SM
Cell fractions			
Control	0.87 ± 0.02	0.64 ± 0.01	0.39 ± 0.01
UV	1.64 ± 0.01	0.34 ± 0.01	0.62 ± 0.02
Relative change	1.89	0.53	1.59
Vesicle fractions			
Control	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
UV	0.46 ± 0.02	0.12 ± 0.01	0.55 ± 0.02
Relative change	5.75	1.50	6.11

CHO-K1 cells were treated with or without UV light and cultured for 24 h in serine-free DMEM with 12 μCi [^{3}H]serine. Cells were collected by centrifugation and lipids were extracted from cell pellets and vesicles as described in Methods. Lipids were separated by TLC and radioactivity in phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn) and sphingomyelin (SM) was normalized relative to total phosphorus. Data are mean ± SEM of 6 samples. Relative changes were expressed as fold increase in UV-treated cells compared to untreated cells using averages from 6 samples.

Figure 20. Lack of effects of z-VAD-fmk on PtdSer and SM biosynthesis and blockage of UV-induced apoptosis in CHO-K1 cells. In panel A, CHO-K1 cells were treated with or without UV light and incubated for 12 h with 100 μM z-VAD-fmk. PARP cleavage was detected as described in Methods. For panels B and C, following treatment of cells with or without UV light, cells were incubated with 12 μCi [^{3}H]serine in serine-free DMEM with or without 100 μM z-VAD-fmk for 12 h. Cells were harvested and lipids were extracted and separated by TLC. Radioactivity in PtdSer or SM was determined. Data are mean ± SEM from 6 samples. Panel B, PtdSer biosynthesis; panel C, SM biosynthesis.

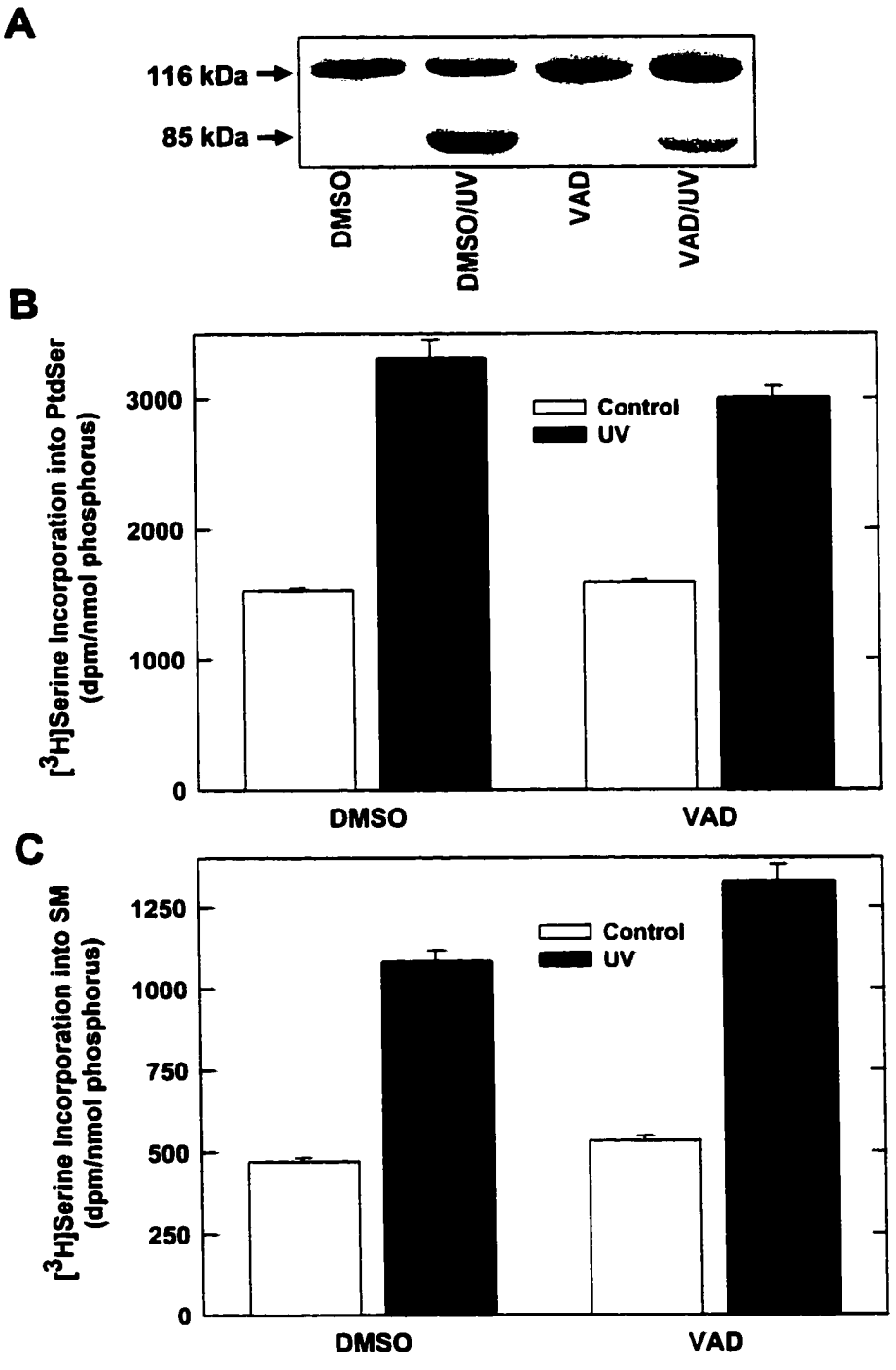
A
116 kDa
85 kDa
DMSO
DMSO/UV
VAD
VAD/UV
B
[3H]Serine Incorporation into PtdSer (dpm/nmol phosphorus)
3000
2000
1000
0
Control
UV
DMSO
VAD
C
[3H]Serine Incorporation into SM (dpm/nmol phosphorus)
1250
1000
750
500
250
0
Control
UV
DMSO
VAD

Figure 20

Figure 21. Expression of Myc-PSS I or II in CHO-K1 cells and detection of serine base-exchange activity *in vitro*. For panel A, CHO-K1 cells were transfected with empty expression vectors, pcDNA-PSS I or pcDNA-PSS II constructs. Cells were harvested 24 h (PSS I) or 48 h (PSS II) after transfection, proteins were extracted and separated (20 μg) by SDS-PAGE. Myc-PSS I and PSS II were detected by Western blotting with anti-c-Myc mAb. For panel B, immunofluorescence detection of c-Myc-tagged proteins was performed on cells stably expressing PSS I or PSS II as described in Methods. Images were obtained using confocal microscopy. Bar, 10 μm. For panel C, cell extracts from PSS I, PSS II cells and control cells were prepared and assayed for serine base-exchange activities as described in Methods. Values are mean ± SEM for 6 samples.

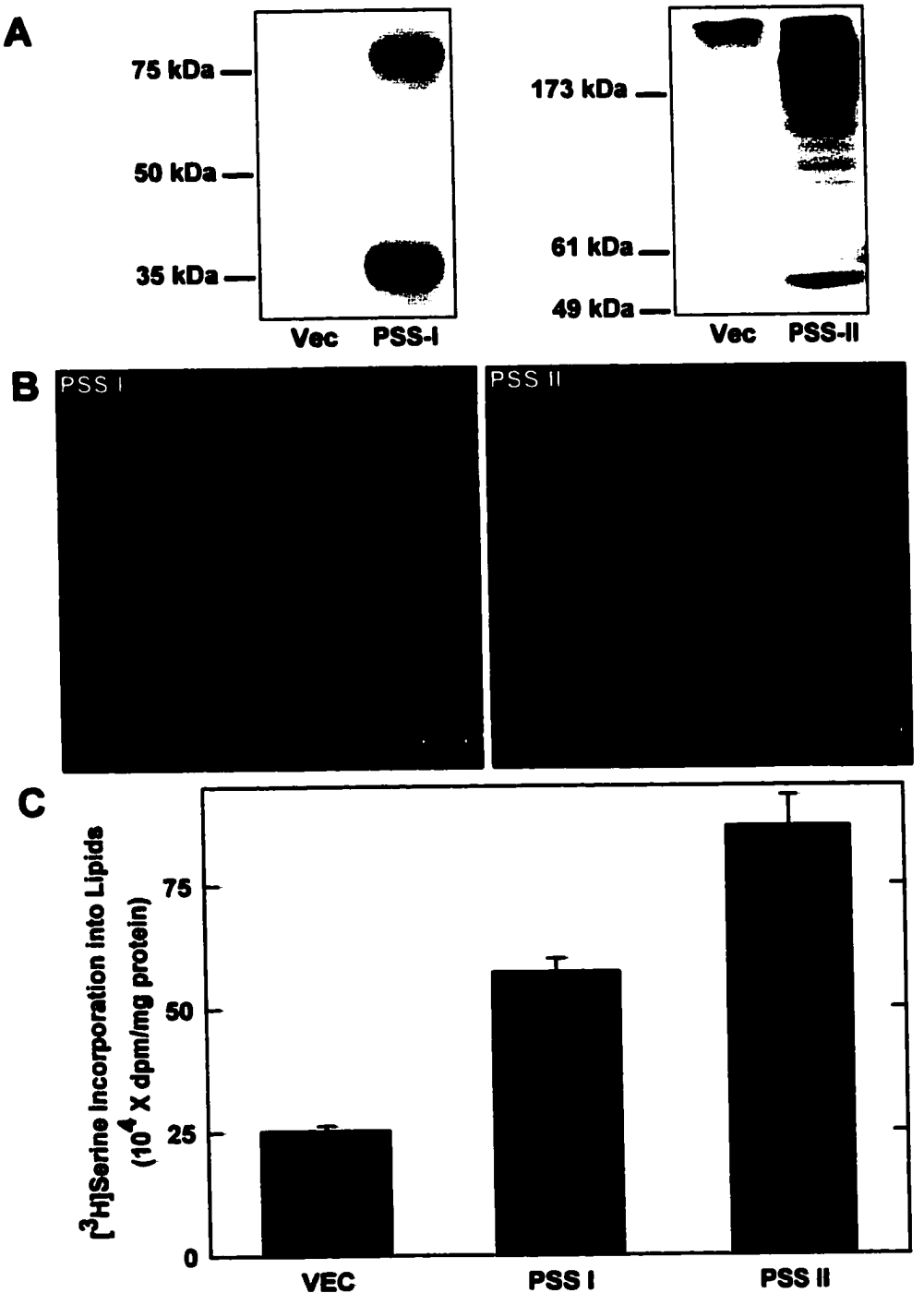
A
75 kDa
50 kDa
35 kDa
Vec
PSS-I
173 kDa
61 kDa
49 kDa
Vec
PSS-II
B
PSS I
PSS II
C
[3H]Serine Incorporation into Lipids
(10^4 X dpm/mg protein)
75
50
25
0
VEC
PSS I
PSS II

Figure 21

Immunofluorescence of these cells with anti-Myc antibody showed that both PSS I and II localized to ER (Fig. 21B). The expression of c-Myc-tagged PSS I was not as stable as that of c-Myc-tagged PSS II, possibly related to modification or hydrolysis at the C-terminus of the protein.

2. Resistance to UV-induced apoptosis in cells expressing PSS I or PSS II

When cells over-expressing PSS I or PSS II were irradiated with UV light, they did not show significant apoptotic morphology, such as cell shrinkage, refractile appearance and rounding compared to UV-treated control cells (Fig. 22), indicating possible resistance towards UV-induced apoptosis as a result of over-expression of PSS I or PSS II. Under control conditions, both PSS I and PSS II cells, as well as the control line, showed little response to an antibody raised against an epitope only appearing in activated caspase-3 (Fig. 23, panels A, C and D). After exposure to UV light, extensive activation of caspase-3 occurred in control cells after 12 h incubation (Fig. 23B), but not in PSS I or PSS II over-expressing cells (Fig. 23, panels D and F). Nuclear condensation and fragmentation developed later in UV-treated control cells (Fig. 24B), whereas relatively normal nuclear morphology remained in most cells expressing high levels of PSS I (Fig. 24D); PSS II cells showed less resistance to apoptotic nuclear changes (Fig. 24F). PSS-mediated-resistance to apoptosis varied with the type of inducing stimuli, as cell death initiated by staurosporine (STS) or farnesol in cells over-expressing PSS I or II was accompanied by little difference in apoptotic morphology compared to treated control cells (Fig. 25).

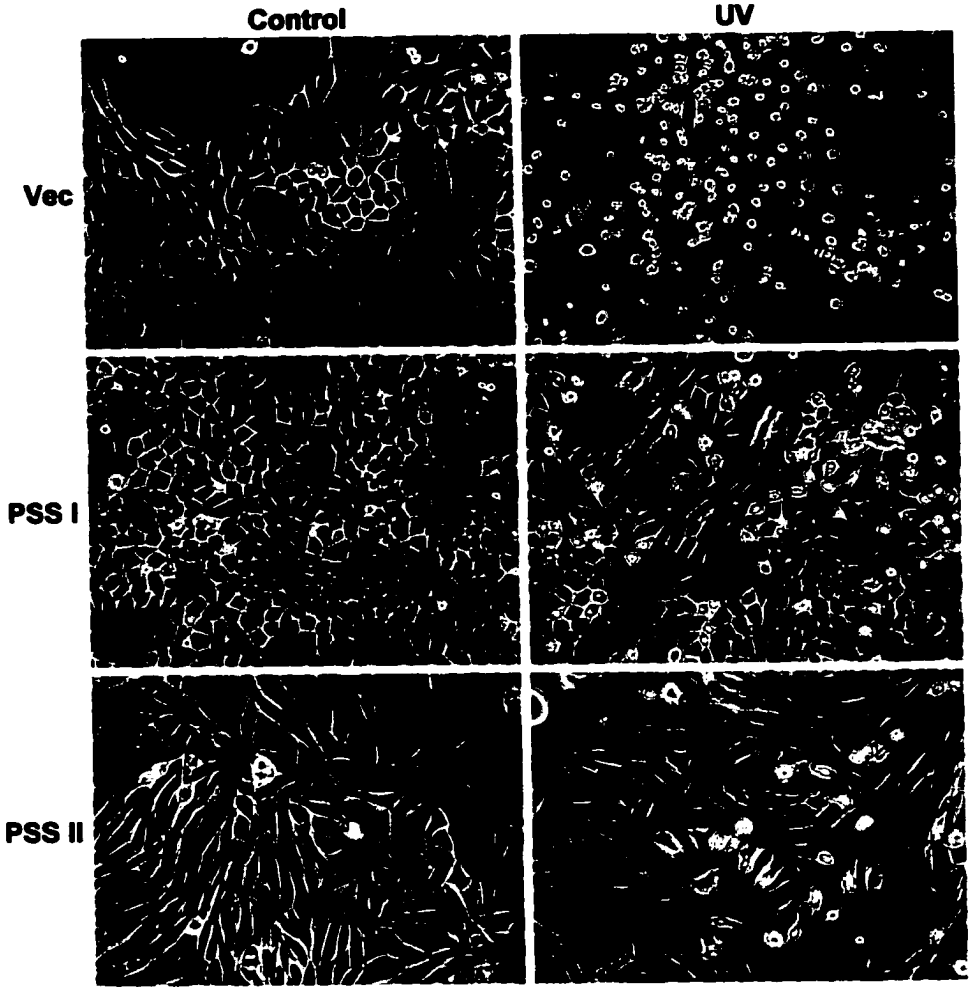

Figure 22. Morphology of CHO-K1 cells over-expressing PSS I or PSS II following UV irradiation. CHO-K1 cells over-expressing PSS I, PSS II and control clones were grown in DMEM and treated without or with UV light. After 12 h of incubation, phase contrast photographs were taken at 300X magnification.

Figure 23. Caspase-3 activation following UV irradiation of CHO-K1 cells over-expressing PSS I or PSS II. CHO-K1 cells over-expressing PSS I, PSS II and control clones were treated with (panel B, D and F) or without (panels A, C and E) UV light and cultured for an additional 12 h. Cells were fixed, permeabilized and incubated with anti-active-caspase-3 pAb followed by anti-c-Myc mAb. Fluorescein-conjugated goat anti-rabbit IgG was used to detect the activated form of caspase-3 (green). Texas Red conjugated goat anti-mouse IgG was used to detect Myc-PSS I or PSS II (red). Panels A and B, vector control cells; panels C and D, PSS I cells; panels E and F, PSS II cells. Bar, 20 μm.

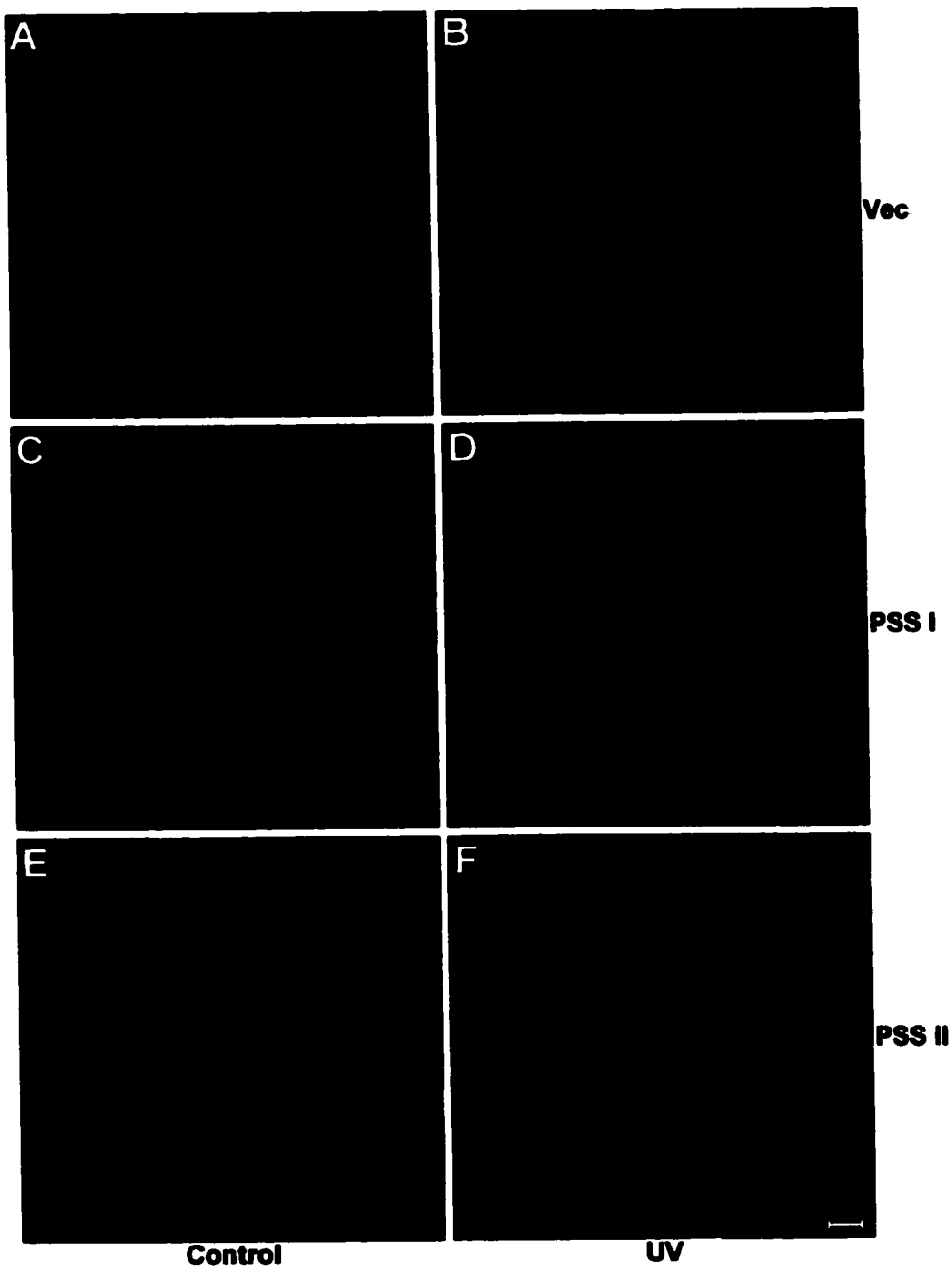

Figure 23

Figure 24. Lack of nuclear changes following UV irradiation in CHO cells over-expressing PSS I or PSS II. CHO-K1 cells over-expressing PSS I, PSS II and control clones were treated with (panels B, D and F) or without (panels A, C and E) UV light and cultured for an additional 16 h. Cells were fixed, permeabilized and incubated with anti-c-Myc mAb. Fluorescein-conjugated goat anti-mouse IgG was used to detect PSS I and PSS II (green). Propidium iodide was used to stain the nucleus (red) as described in Methods. Panels A and B, vector cells; panels C and D, PSS I cells; panels E and F, PSS II cells. Bar, 10 μm.

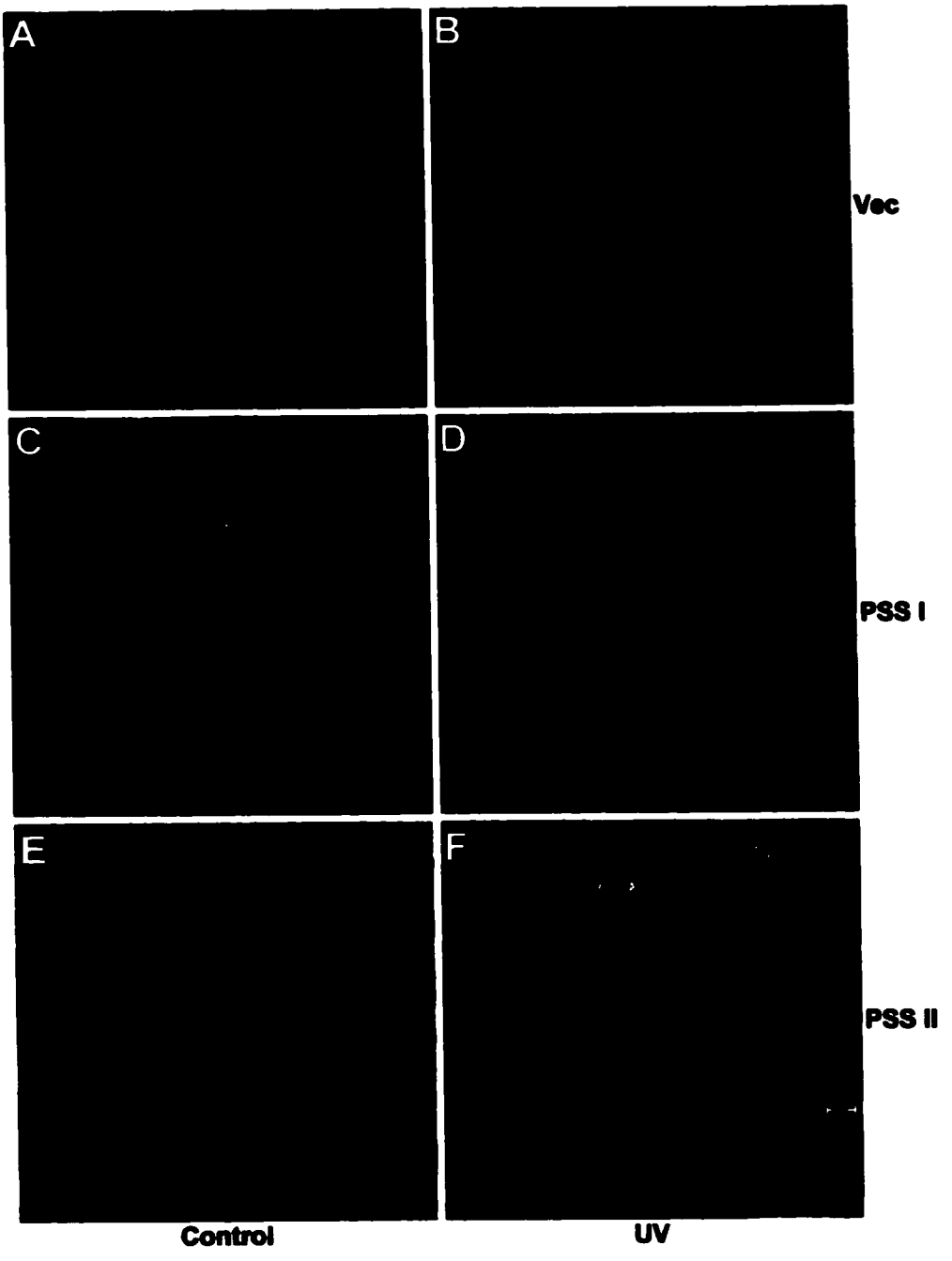

Figure 24

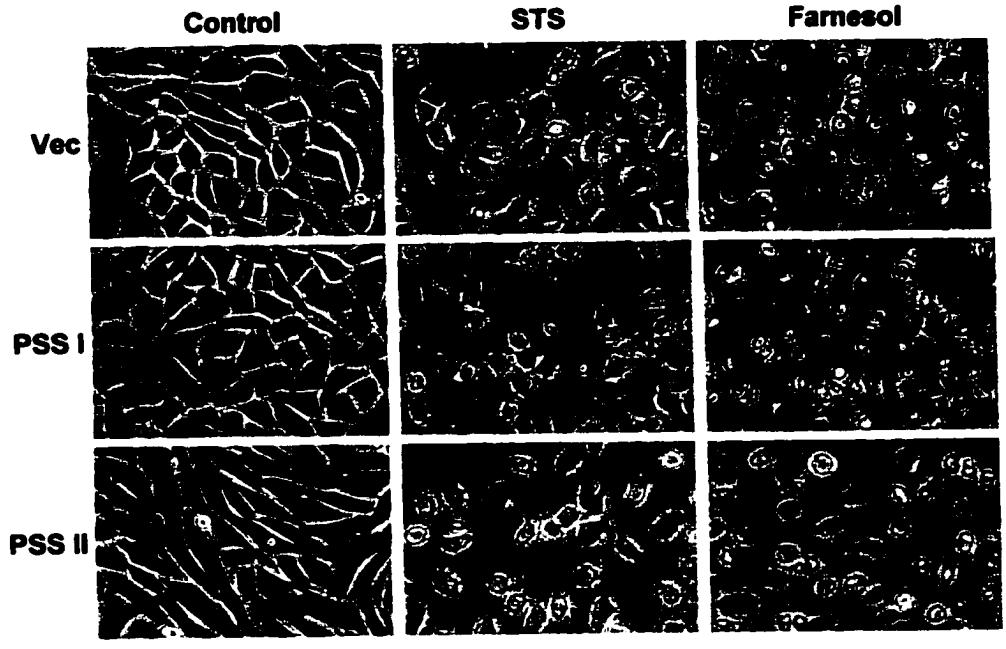

Figure 25. Morphology of CHO-K1 cells over-expressing PSS I or PSS II following staurosporine or farnesol treatment. CHO-K1 cells over-expressing PSS I, PSS II and control clones were grown in DMEM and treated with STS (0.5 μM) for 4 h or with farnesol (30 μM) for 3 h. Phase contrast photographs were taken at 300X magnification.

3. Stimulation of PtdSer synthesis in cells over-expressing PSS I or PSS II following UV irradiation

To test the involvement of PSS I and PSS II in regulating PtdSer formation during UV-induced apoptosis, CHO cells over-expressing the two enzymes were exposed to UV light and synthesis of serine-derived phospholipids, including PtdSer, SM, and PtdEtn from PtdSer decarboxylation, was monitored using [^{3}H]serine. PtdSer accounted for ~70% of the labeled phospholipids, with PtdEtn (~15%) and SM (~10%) accounting for most of the remainder. Cells over-expressing PSS I had a slightly higher basal rate of PtdSer formation and ~50% higher PtdSer decarboxylation to PtdEtn compared to control cells, whereas no major difference in SM synthesis was observed (Fig. 26). When irradiated by UV exposure, PSS I cells showed earlier stimulation of PtdSer biosynthesis with PtdSer levels rising above those of UV-treated control cells by 3 h; the increase in UV-treated control cells started around 8 h after UV irradiation when apoptotic morphology started to develop (Fig. 26, upper panel). After 24 h of incubation following UV irradiation, PtdSer levels in UV-treated PSS I cells remained 1.5-2 fold higher than those in UV-treated control cells. Similar to the 50% inhibition of PtdEtn formation observed in UV-treated control cells relative to their untreated counterparts, UV-treated PSS I-expressing cells also had a 50% decrease in PtdSer decarboxylation compared to untreated PSS I cells (Fig. 26, middle panel). PSS I over-expression did not change SM biosynthesis relative to control cells under control conditions whereas UV-treated PSS I expressing cells had a lightly higher rate of SM stimulation compared with UV-treated control cells (Fig. 26, bottom panel).

Figure 26. Biosynthesis of serine-derived phospholipids in CHO cells over-expressing PSS I. PSS I (triangles) and control (circles) cells were grown in serine-free DMEM. Following treatment of cells with (solid symbols) or without (open symbols) UV light, the cells were incubated for the periods of time indicated with 20 μCi [^{3}H]serine. Cells were harvested and lipids were extracted from cells and apoptotic vesicles and separated by TLC. Radioactivity in PtdSer, PtdEtn and SM was determined as described in Methods. The results are mean ± SEM of 6 samples.

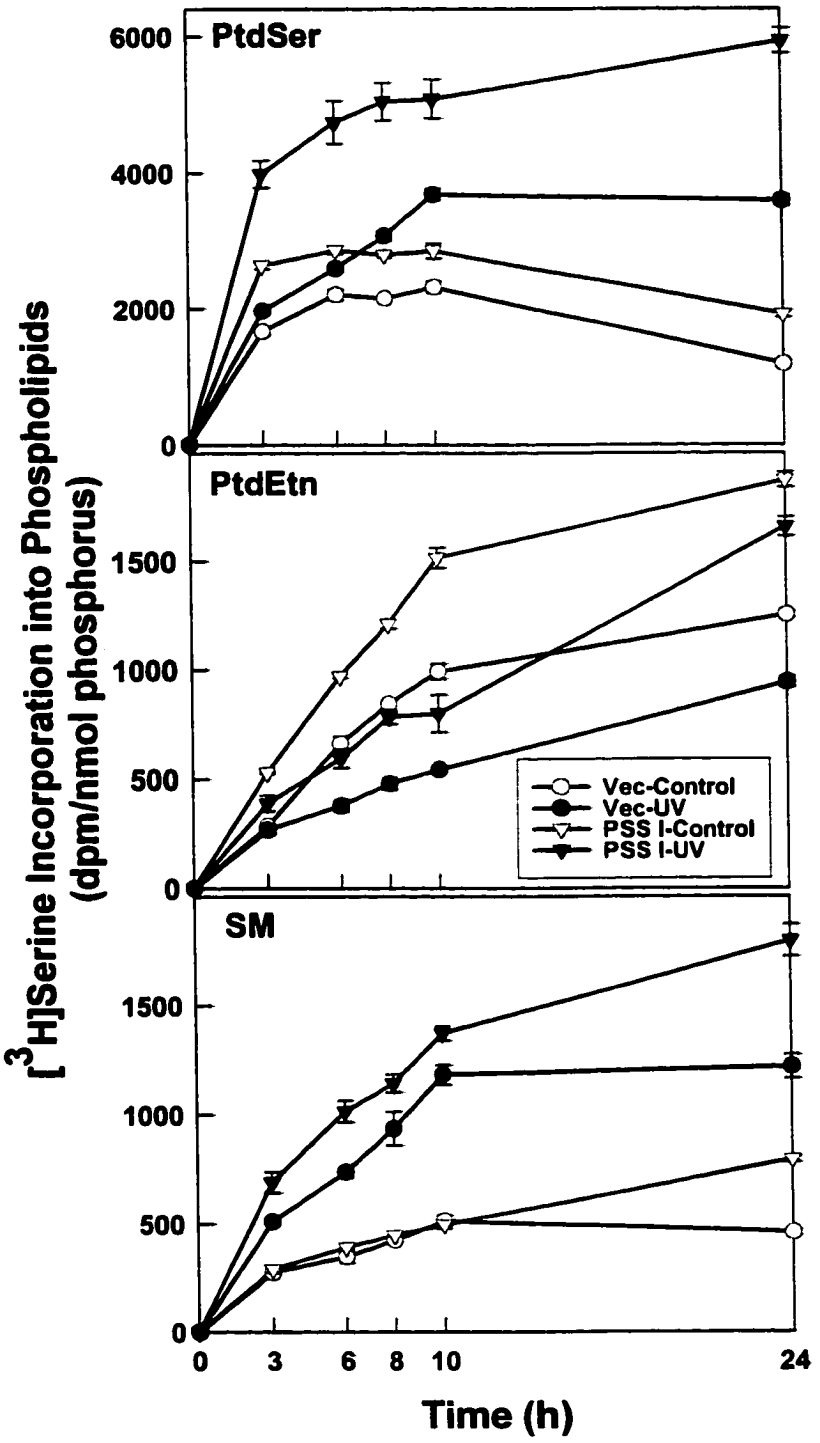
PtdSer
PtdEtn
SM
6000
4000
2000
0
1500
1000
500
0
1500
1000
500
0
0
3
6
8
10
24
Time (h)
[3H]Serine Incorporation into Phospholipids (dpm/nmol phosphorus)
Vec-Control
Vec-UV
PSS I-Control
PSS I-UV

Figure 26

When PSS II was over-expressed in CHO-K1 cells, a higher basal level of PtdSer synthesis (1.5-fold) and decarboxylation (2-fold) were observed whereas SM levels were slightly lowered in cells over-expressing PSS II (Fig. 27). UV irradiation induced significant stimulation of PtdSer formation in PSS II-expressing cells; at all incubation times, PtdSer levels in UV-treated cells expressing PSS II were 2-3 fold higher than those in UV-treated control cells (Fig. 27, upper panel). PtdSer decarboxylation was not changed appreciably in PSS II-expressing or control cells following UV irradiation except at 12 h of incubation, UV-treated cells with PSS II expression had a 70% decrease in PtdEtn formation compared to untreated PSS II-expressing cells (Fig. 27, middle panel). Although SM synthesis increased 3-fold in both UV-treated PSS II and control cells, PSS II-expressing cells showed a 60% lower level of the stimulation compared to control cells (Fig. 27, bottom panel).

Overall, cells expressing PSS I or PSS II showed significant resistance toward UV-induced apoptosis even though PtdSer biosynthesis was enhanced following UV treatment in these cells. Thus, UV irradiation seemed to up-regulate the activity of both PSS I and PSS II, promoting serine base-exchange reactions to form PtdSer without changing the rate of PtdSer decarboxylation appreciably.

4. Caspase-independent PtdSer synthesis in cells over-expressing PSS I or PSS II

Stimulation of PtdSer biosynthesis in apoptotic CHO-K1 cells was not inhibited by caspase inhibitor, z-VAD-fmk (Fig. 28B). Potential caspase dependency of PtdSer biosynthesis in PSS I and PSS II expressing cells was further studied by co-incubating cells with z-VAD-fmk following UV irradiation. Under these conditions, further stimulation of PtdSer biosynthesis mediated by PSS I and PSS II over-expression was

Figure 27. Biosynthesis of serine-derived phospholipids in CHO cells over-expressing PSS II. PSS II (squares) and control (circles) cells were grown in serine-free DMEM. Following treatment of cells with (solid symbols) or without (open symbols) UV light, the cells were incubated for the periods of time indicated with 20 μCi [^{3}H]serine. Cells were harvested, lipids were extracted from cells and apoptotic vesicles and separated by TLC. Radioactivity in PtdSer, PtdEtn and SM was determined. The results are mean ± SEM of 6 samples.

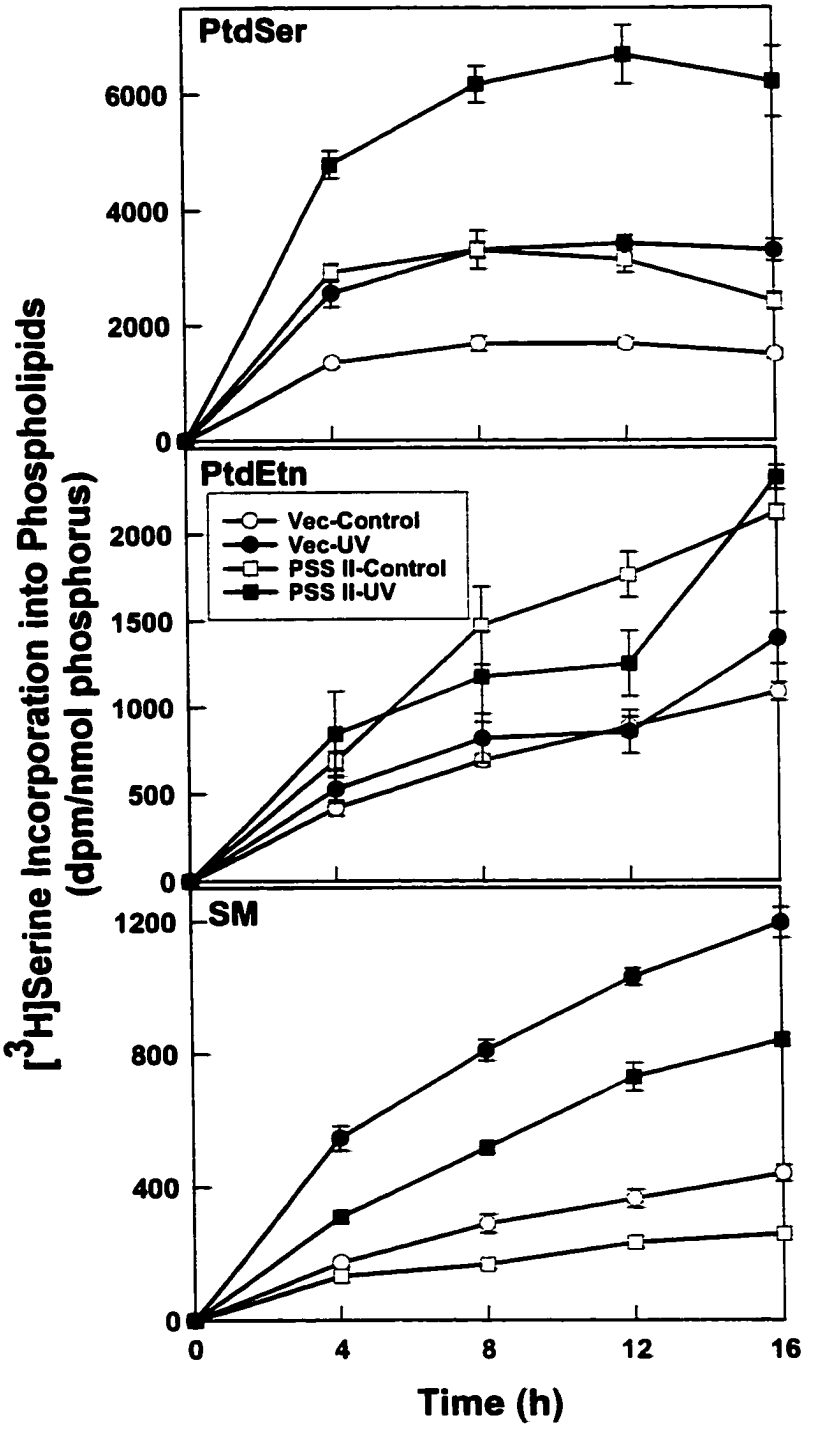
PtdSer
PtdEtn
SM
Vec-Control
Vec-UV
PSS II-Control
PSS II-UV
[3H]Serine Incorporation into Phospholipids (dpm/nmol phosphorus)
Time (h)
0
2000
4000
6000
500
1000
1500
400
800
1200
4
8
12
16

Figure 27

completely insensitive to z-VAD-fmk, although PARP cleavage was blocked (Fig. 28, panels A and B). PtdEtn formation from PtdSer decarboxylation was stimulated in cells over-expressing PSS I or PSS II whereas the level of stimulation was reduced following UV irradiation in both cases. These changes were not affected by the inhibition of caspase activation by z-VAD-fmk (Fig. 28C). Over-expression of PSS I or PSS II resulted in little changes in SM biosynthesis compared to control cells with or without the exposure to UV, and the presence of z-VAD-fmk had no appreciable effects (Fig. 28D).

5. PtdCho and PtdEtn synthesis in cells over-expressing PSS I or PSS II

PtdCho biosynthesis from CDP-choline pathway was monitored with [^{3}H]choline. Over-expression of PSS I and II did not change the rate of PtdCho biosynthesis, and UV irradiation slightly decreased PtdCho biosynthesis in control cells (Table 3). PtdEtn formation from CDP-ethanolamine pathway is not the main source of PtdEtn in CHO cells (Shiao et al., 1995; Voelker, 1984). Low levels of [^{14}C]ethanolamine incorporation were observed in CHO-K1 cells (Table 3). PSS I expressing cells showed the same rate of PtdEtn synthesis as the control cells and UV treatment had no appreciable effects.

D. Phospholipid scramblase-mediated PtdSer biosynthesis during apoptosis

1. Stable expression of murine PLSCR1 and PLSCR2 in CHO-K1 cells

C-Myc-tagged PLSCR1 (328 amino acids) and PLSCR2 (307 amino acids) were transiently expressed in CHO-K1 cells and over-expressed proteins were detected with anti-c-Myc mAb. Both PLSCR isoforms migrated with predicted molecular weights (Fig. 29A). Clones stably over-expressing PLSCR1 and PLSCR2 were then selected. Initially, selection using G418 alone failed to produce positive clones, raising the possibility that cells over-expressing PLSCR were outgrown by cells where only the neomycin resistance

Figure 28. Effects of z-VAD-fmk on PARP cleavage and phospholipid biosynthesis following UV irradiation of CHO-K1 cells over-expressing PSS I or PSS II. For panel A, cells were treated with or without UV light and incubated for 12 h with 100 μM z-VAD-fmk. Proteins were extracted and PARP cleavage was detected. For panels B, C and D, PSS I, PSS II and control cells were grown in serine-free DMEM. Following treatment of cells with or without UV light, 100 μM z-VAD-fmk was added to cells as indicated. Cells were incubated for 8 h (PSS I cells) or 12 h (PSS II cells) in the presence of 20 μCi of [^{3}H]serine. Cells were harvested and lipid extraction, phospholipids separation and quantitation were performed. Radioactivity in individual phospholipids was determined. Data are mean ± SEM for 6 samples. Panel B, PtdSer biosynthesis; Panel C, PtdEtn biosynthesis; Panel D, SM biosynthesis.

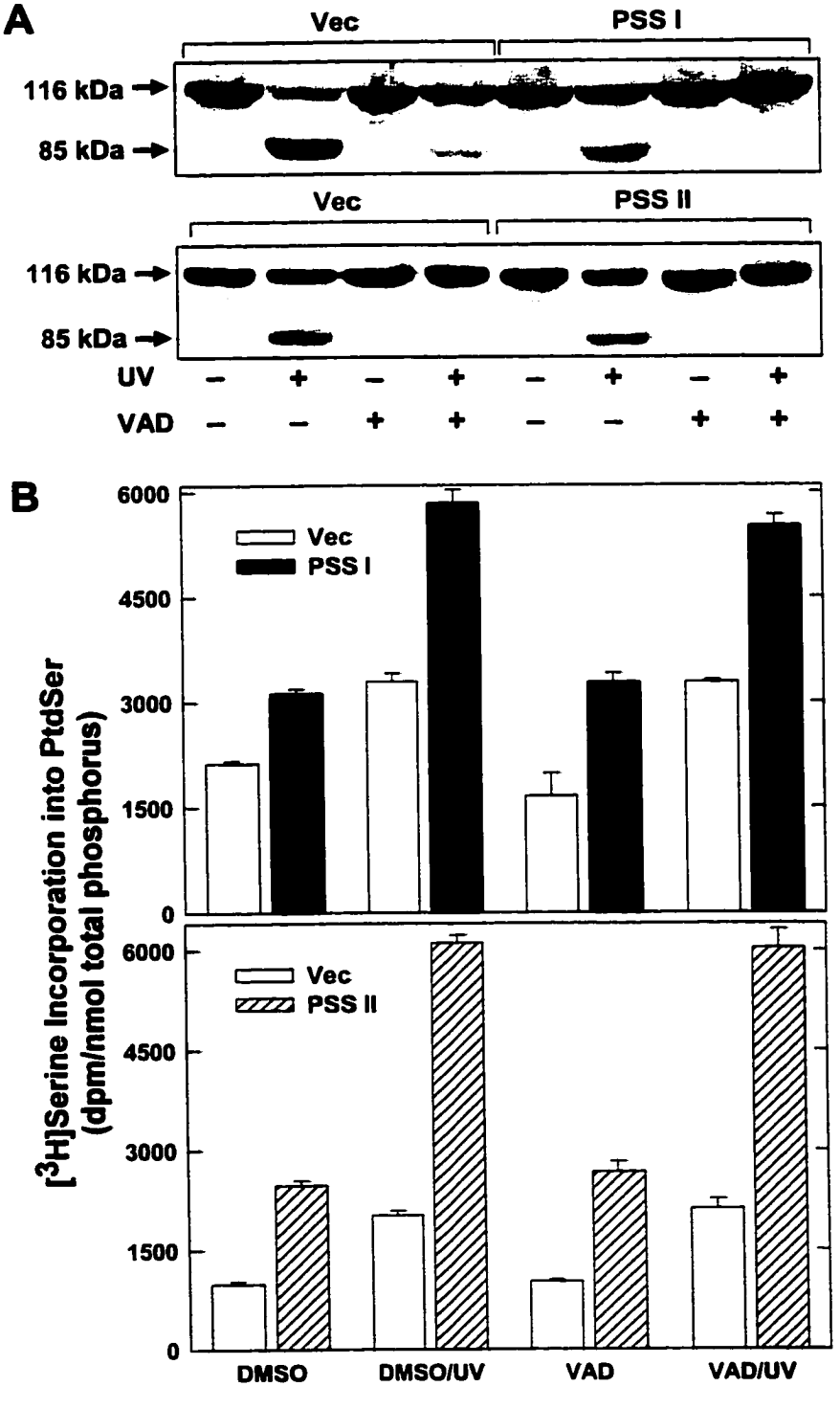
A
Vec
PSS I
116 kDa
85 kDa
Vec
PSS II
116 kDa
85 kDa
UV
VAD
B
[3H]Serine Incorporation into PtdSer (dpm/nmol total phosphorus)
Vec
PSS I
Vec
PSS II
6000
4500
3000
1500
0
DMSO
DMSO/UV
VAD
VAD/UV

Figure 28

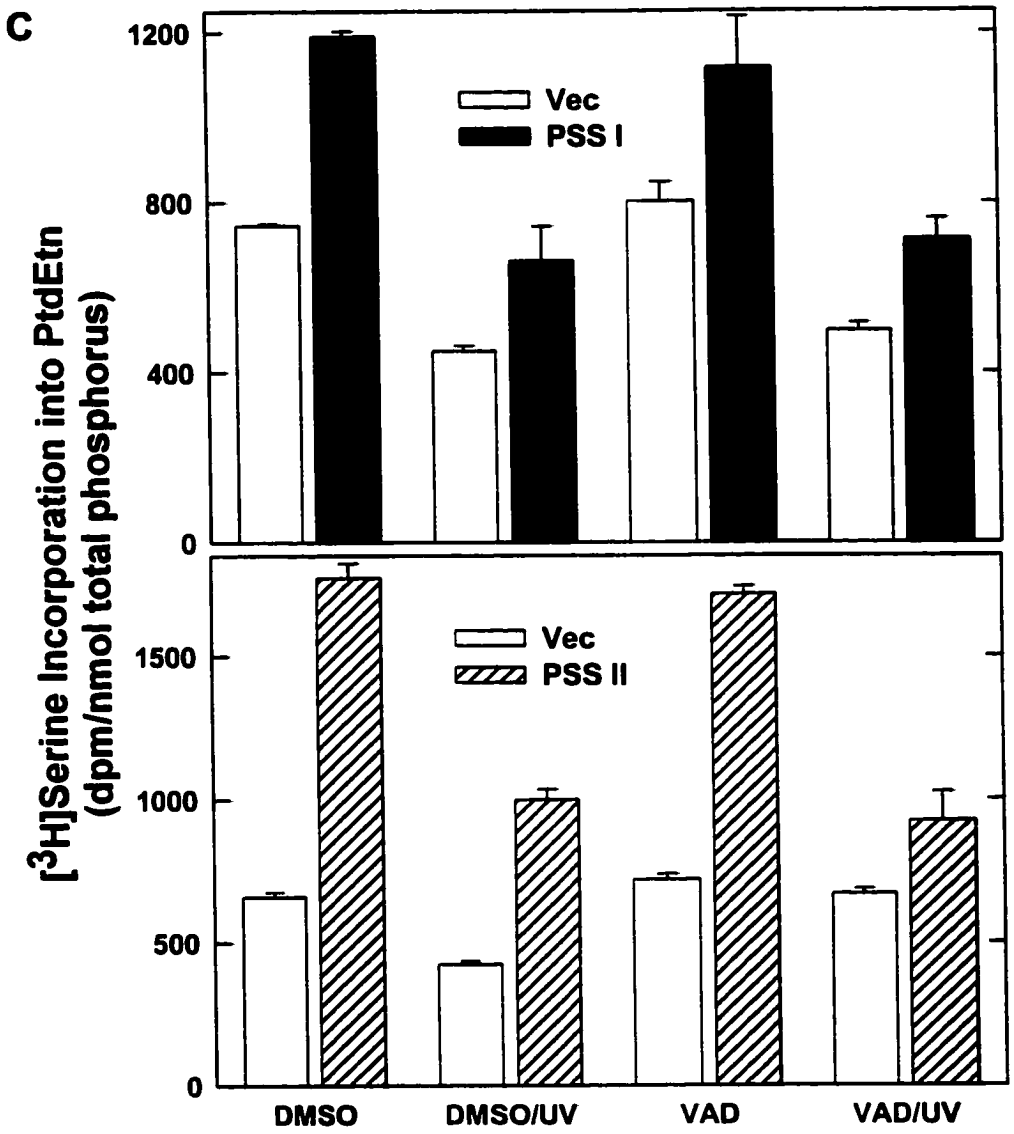
C
[3H]Serine Incorporation into PtdEtn
(dpm/nmol total phosphorus)
1200
800
400
0
Vec
PSS I
1500
1000
500
0
Vec
PSS II
DMSO
DMSO/UV
VAD
VAD/UV

Figure 28

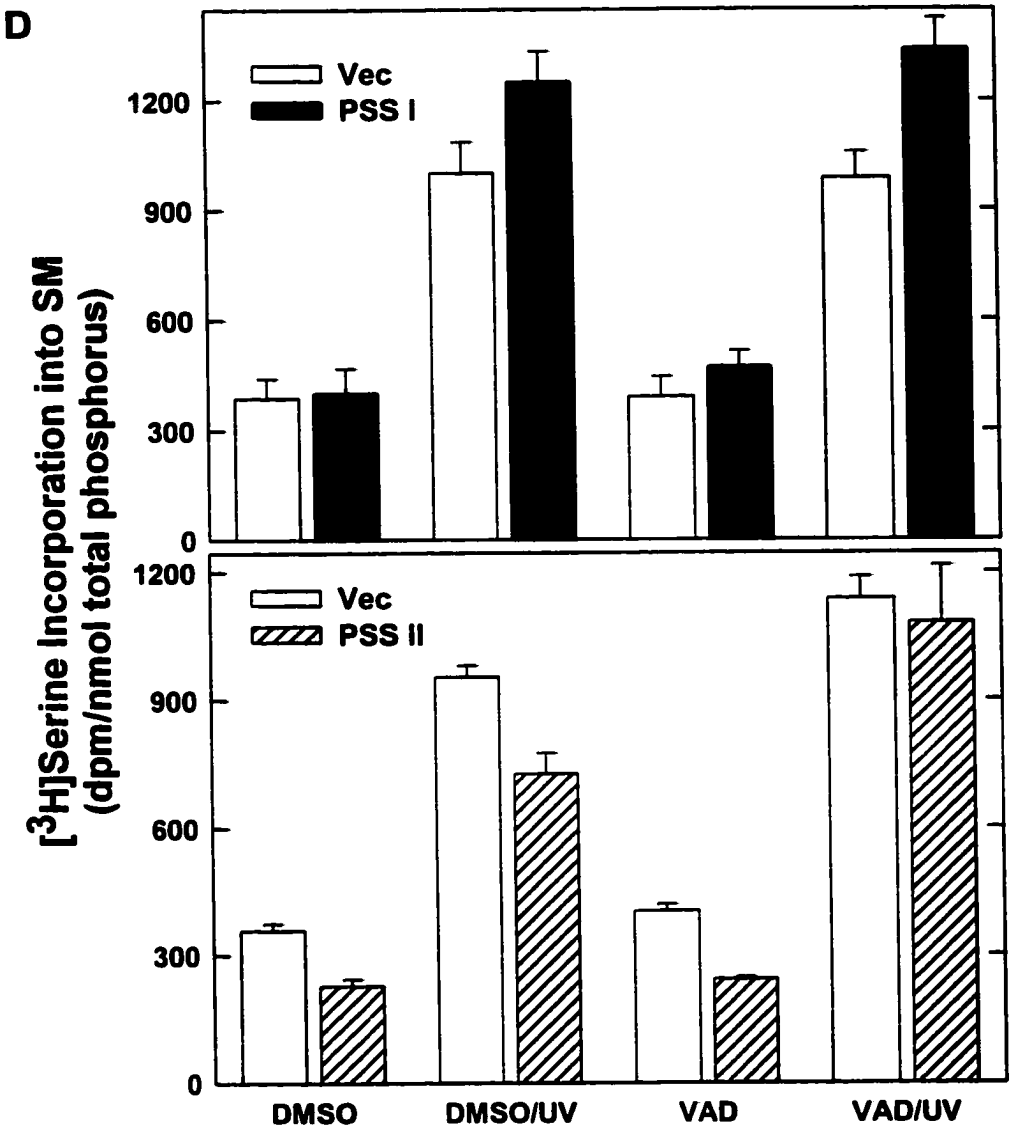
D
Vec
PSS I
Vec
PSS II
[3H]Serine Incorporation into SM
(dpm/nmol total phosphorus)
0
300
600
900
1200
DMSO
DMSO/UV
VAD
VAD/UV

Figure 28

Table 3. PtdCho and PtdEtn biosynthesis in cells over-expressing PSS I or PSS II through CDP-choline and CDP-ethanolamine pathways

	PtdCho	PtdEtn
	[^{3}H]choline incorporation (1×10^4 dpm/nmol phosphorus)	[^{14}C]ethanolamine incorporation (1×10^3 dpm/nmol phosphorus)
Vector cells		
Control	2.75 ± 0.05	1.02 ± 0.04
UV	2.13 ± 0.07*	0.94 ± 0.03
PSS I cells		
Control	2.46 ± 0.08	1.01 ± 0.00
UV	2.13 ± 0.02	0.94 ± 0.02*
Vector cells		
Control	4.08 ± 0.07	NT
UV	3.67 ± 0.09*	NT
PSS II cells		
Control	4.11 ± 0.10	NT
UV	4.02 ± 0.05	NT

* $p < 0.05$ versus control
NT not tested

CHO-K1 clones over-expressing PSS I or PSS II and control cells were grown in choline-free DMEM. Following treatment without or with UV light for 10 min, [^{3}H]choline or [^{14}C]ethanolamine (3 μCi) was added to cells. Cells were incubated for 8 h (PSS I cells) or 12 h (PSS II cells) and harvested as described in Methods; lipids were extracted and were separated by TLC. Radioactivity in PtdCho or PtdEtn was determined. Data are mean ± ½ range or SEM of 2-4 samples. The level of significance was assessed with a Student's *t*- test for unpaired data.

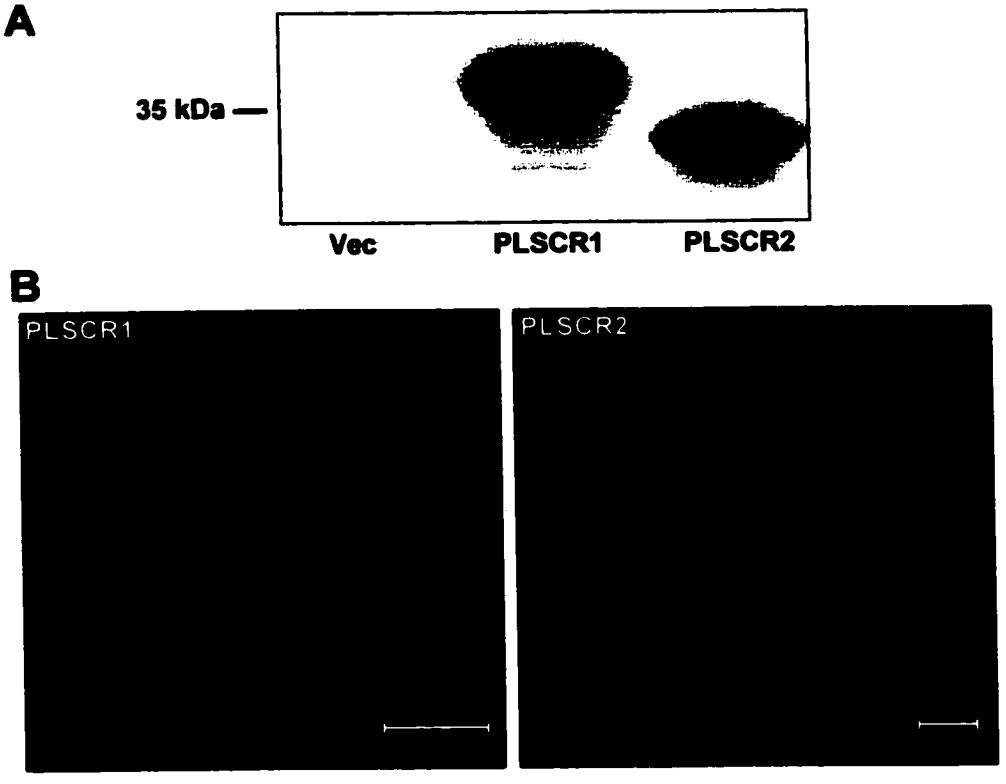

Figure 29. Expression of c-Myc-tagged PLSCR1 or PLSCR2 in CHO-K1 cells. Panel A, CHO-K1 cells were transfected with empty expression vector, pcDNA-PLSCR1 or pcDNA-PLSCR2 constructs. Cells were harvested 24 h after transfection and proteins were extracted and separated (20 μg) using SDS-PAGE and transferred to PVDF membrane. C-Myc-tagged proteins were detected by Western blotting with anti-c-Myc mAb. Panel B, immunofluorescence was performed on CHO-K1 clones stably over-expressing PLSCR1 and PLSCR2. Cells were fixed, permeabilized and incubated with anti-c-Myc mAb. Fluorescein-conjugated goat anti-mouse IgG was used to detect Myc-tagged proteins. Bar, 20μm.

gene was incorporated. We then used a strategy to select G418 and hygromycin resistant cells by co-transfecting cells with PLSCR containing vector and an empty vector with a hygromycin resistant gene. With this approach, cell lines stably expressing PLSCR1 or PLSCR2 were produced. Immunofluorescence analyses of these cells showed a predominantly plasma membrane localization of PLSCR1 cells (Fig. 29B, left panel); in some cells, highly expressed PLSCR1 also was seen in the cell nucleus. The shape of PLSCR1 expressing cells was significantly different from that of wild type CHO-K1 cells, the former being rounder and smaller (Fig. 30C). Cells over-expressing PLSCR1 grew at a slower rate, the basal level of cell death was higher and membrane vesiculation was more prominent (Fig. 29B; left panel). Stably expressed PLSCR2 was found predominantly in the cell nucleus, but also was apparent in the cytoplasm (Fig. 29B; right panel). PLSCR2 cells were larger than wild type CHO-K1 cells but had a similar growth rate and basal level of cell death (Fig. 30E).

2. PLSCR1 over-expression facilitated UV-induced apoptosis in CHO-K1 cells

When apoptosis was induced in cells over-expressing PLSCR1 or PLSCR2 by exposing them to UV light for 10 min, PLSCR1 cells showed earlier signs of apoptotic morphology (Fig. 30D). Six hours after UV irradiation, when a majority of control cells with empty vector showed little signs of apoptosis (Fig. 30B), the PLSCR1 cells became strongly light-reflecting and cell shrinkage was obvious. PLSCR2 cells did not show major differences in cell morphology compared to control cells (Fig. 30, panels E and F). To detect pro-caspase-3 activation as a marker for the progression of apoptosis, antibody that recognized only active caspase-3 was used to detect caspase activation in PLSCR1 and control cells (Fig. 31). Extensive activation of caspase-3 was observed in PLSCR1 cells while only a few control cells showed positive signals of activated caspase-3 after same period of incubation following UV exposure. Only slight differences, mainly related to increased cell size and not increased caspase-3 activation, were observed with

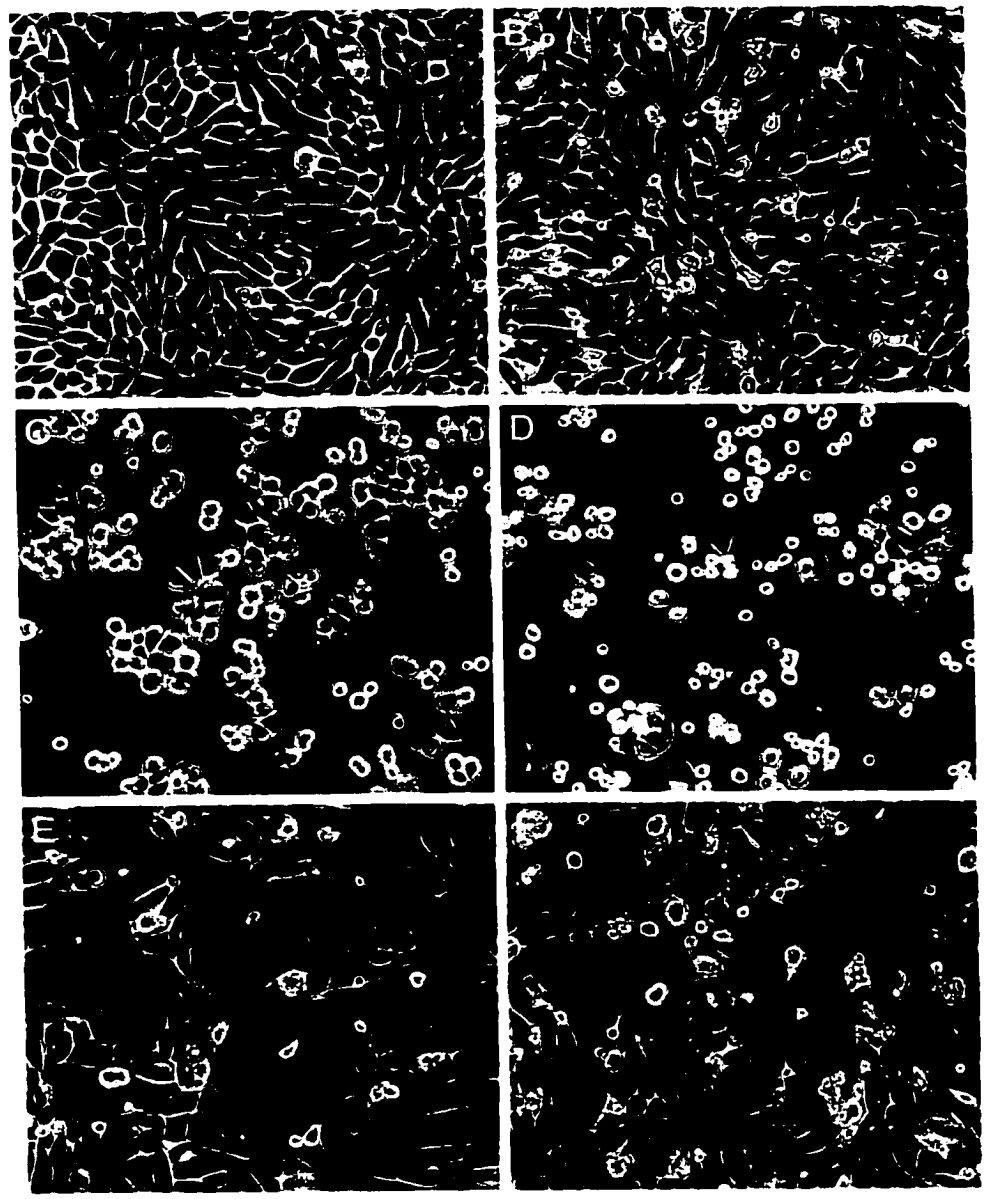

Figure 30. Early occurrence of apoptosis in CHO-K1 cells over-expressing PLSCR1 following UV irradiation. CHO-K1 cells expressing PLSCR1, PLSCR2 and control clones were treated without (panels A, C, E) or with (panels B, D, F) UV light. After 6 h of incubation, phase contrast photographs were taken at 300X magnification. Panels A and B, control cells; panels C and D, PLSCR1 cells; panels E and F, PLSCR2 cells.

Figure 31. Early caspase-3 activation following UV irradiation in CHO-K1 cells over-expressing PLSCR1. CHO-K1 cells over-expressing PLSCR1, PLSCR2 and control clones were treated without (panels A, C, E) or with (panels B, D, F) UV light and were cultured for 6 h. Cells were fixed, permeabilized and incubated with anti-active-caspase-3 pAb. Fluorescein-conjugated goat anti-rabbit IgG was used to detect the activated form of caspase-3 (green). For panels E and F, cells were also incubated with anti-c-Myc mAb, Texas Red-conjugated goat anti-mouse IgG was used to detect PLSCR2 (red). Panels A and B, control cells; panels C and D, PLSCR1 cells; panels E and F, PLSCR2 cells. Bar, 20 μm.

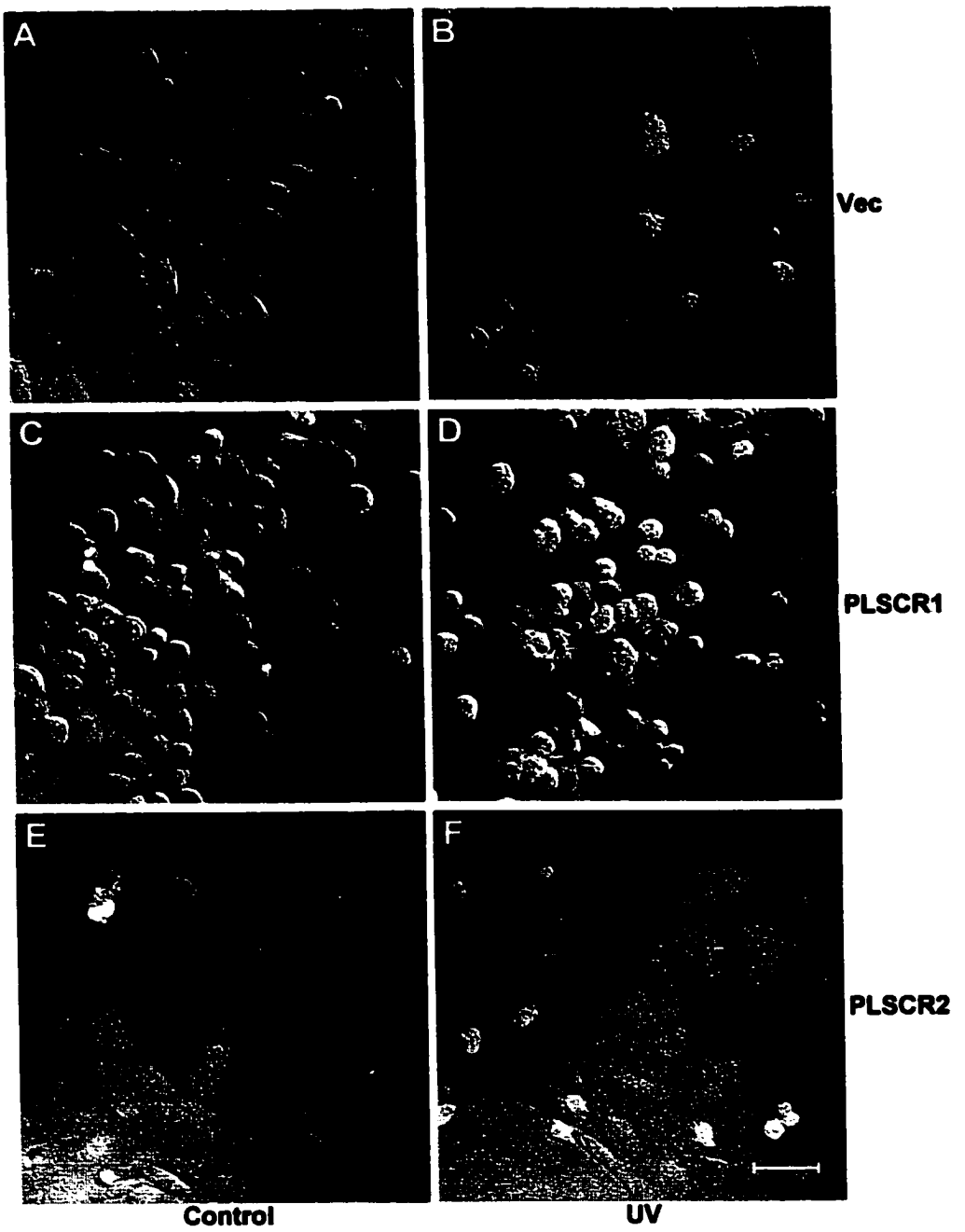

Figure 31

PLSCR2 cells compared to controls. Extensive nuclear condensation and fragmentation were observed in PLSCR1 cells following 8 h incubation after UV irradiation (Fig. 32), whereas a similar extent of apoptotic nuclear changes appeared 4 h later in control cells.

3. PtdSer exposure in PLSCR1 cells induced with UV irradiation

Although PLSCR1 is a key enzyme in phospholipid scrambling, over-expression of PLSCR1 in CHO-K1 cells resulted in only a slightly higher basal level of PtdSer externalization without UV irradiation. PtdSer on the cell surface remained low for control cells at all times. In contrast, when apoptosis was induced with UV irradiation, PtdSer levels on the surface of PLSCR1 cells increased as early as 3 h after treatment, whereas UV-treated control cells showed little change until 8 h (Fig. 33A). PtdSer externalization increased dramatically in PLSCR1 cells 6 h after UV treatment, and by 8 h the majority of PLSCR1 cells were stained with annexin-V-FITC, indicating major PtdSer externalization (Fig. 33B). Integrity of cells was maintained and few cells were stained with propidium iodide in their nuclei. PLSCR2 cells did not show major differences in exposure of PtdSer compared to control cells (Fig. 34). Thus, PLSCR1 over-expression facilitated apoptosis and increased PtdSer externalization in CHO-K1 cells after UV irradiation, whereas PLSCR2 over-expression did not appear to enhance PtdSer externalization or apoptosis.

4. Stimulation of PtdSer biosynthesis in PLSCR1 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 (Yu et al., 2000). Our preliminary experiments indicated that similar stimulation of PtdSer biosynthesis also was observed in CHO-K1 cells during UV-

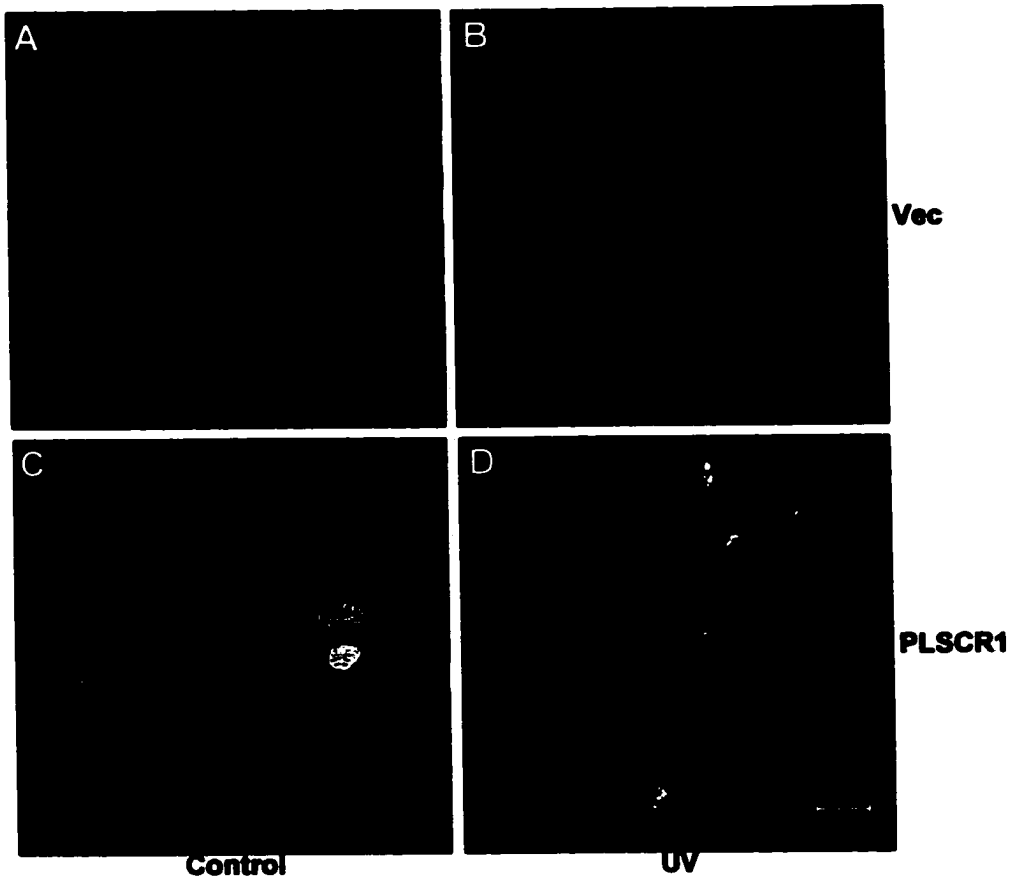

Figure 32. Nuclear changes following UV irradiation in CHO-K1 cells over-expressing PLSCR1. PLSCR1-expressing and control cells were treated without (panels A and C) or with (panels B and D) UV light and cultured for 8 h. Cells were fixed, permeabilized and incubated with anti-c-Myc mAb. Fluorescein-conjugated goat anti-mouse IgG was used to detect PLSCR1 (green). Propidium iodide was used to stain the nucleus (red). Panels A and B, vector control cells; panels C and D, PLSCR1 cells. Bar, 20 μm.

Figure 33. Increase in PtdSer externalization following UV irradiation in CHO-K1 cells over-expressing PLSCR1. CHO-K1 cells over-expressing PLSCR1 (panel B) and vector control cells (panel A) were treated without or with UV light; cells were further cultured for different periods of time. FITC-annexin-V (green) and propidium iodide (red) binding assays were performed as described in Methods. Bar, 20 μm.

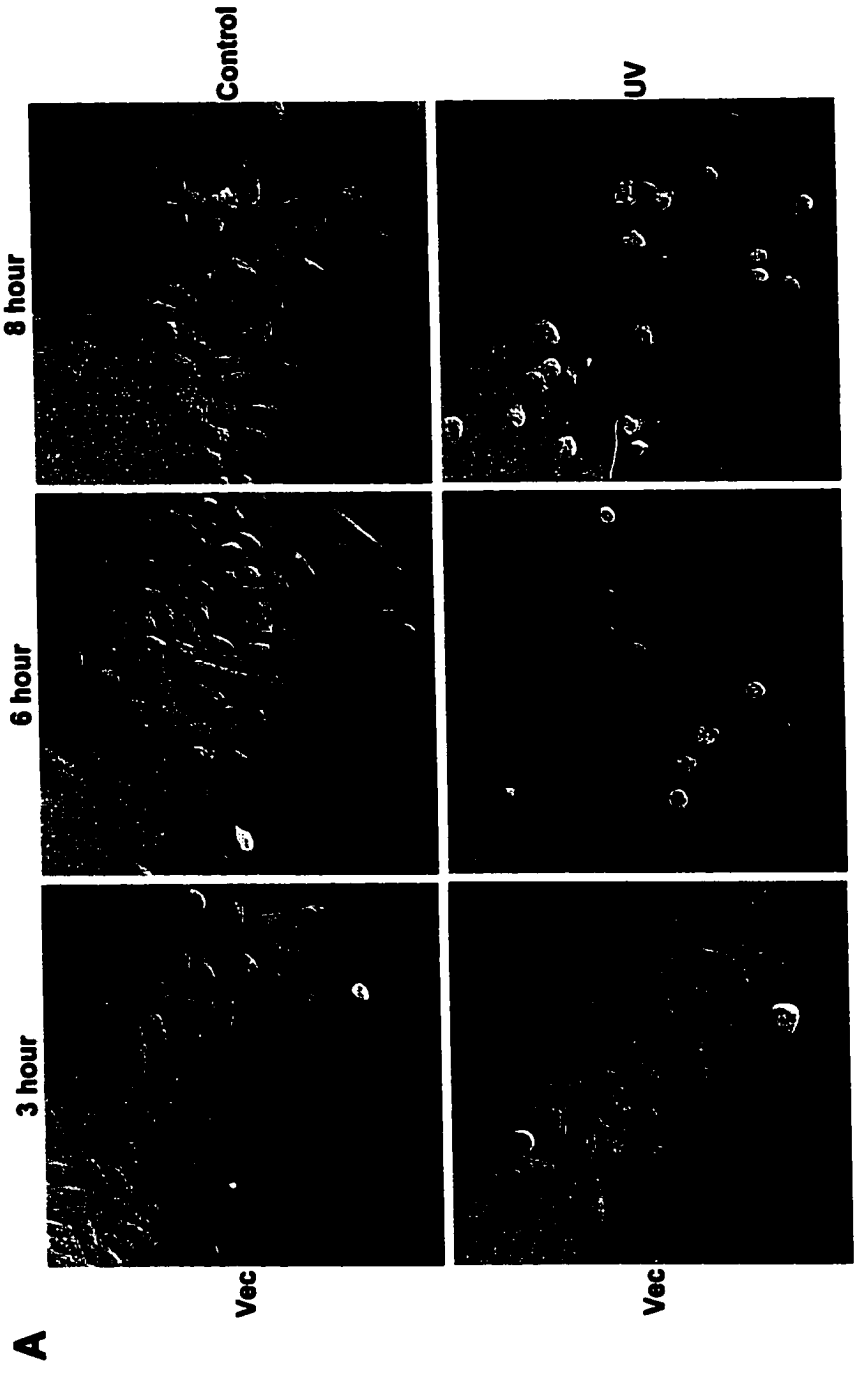

Figure 33

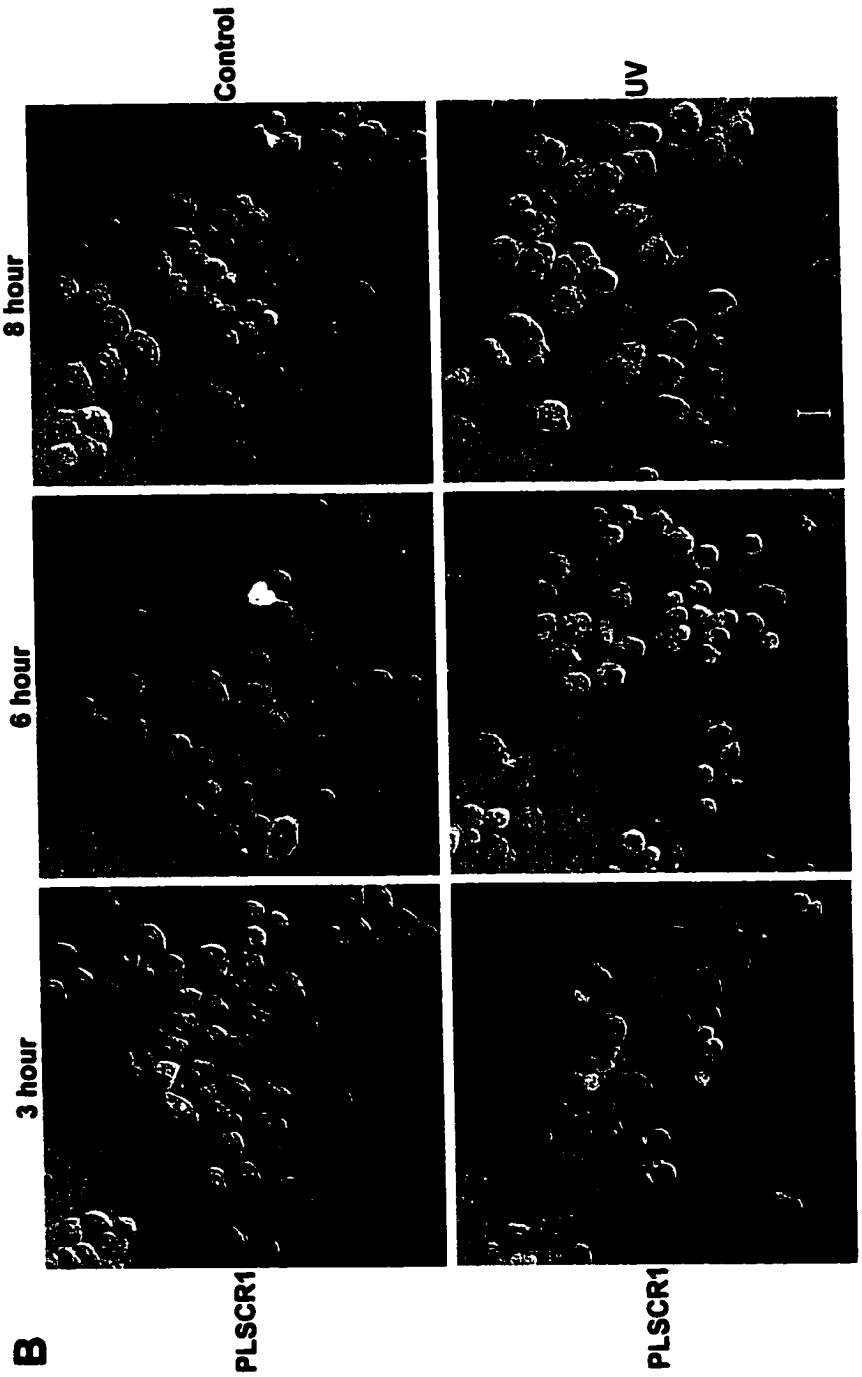

Figure 33

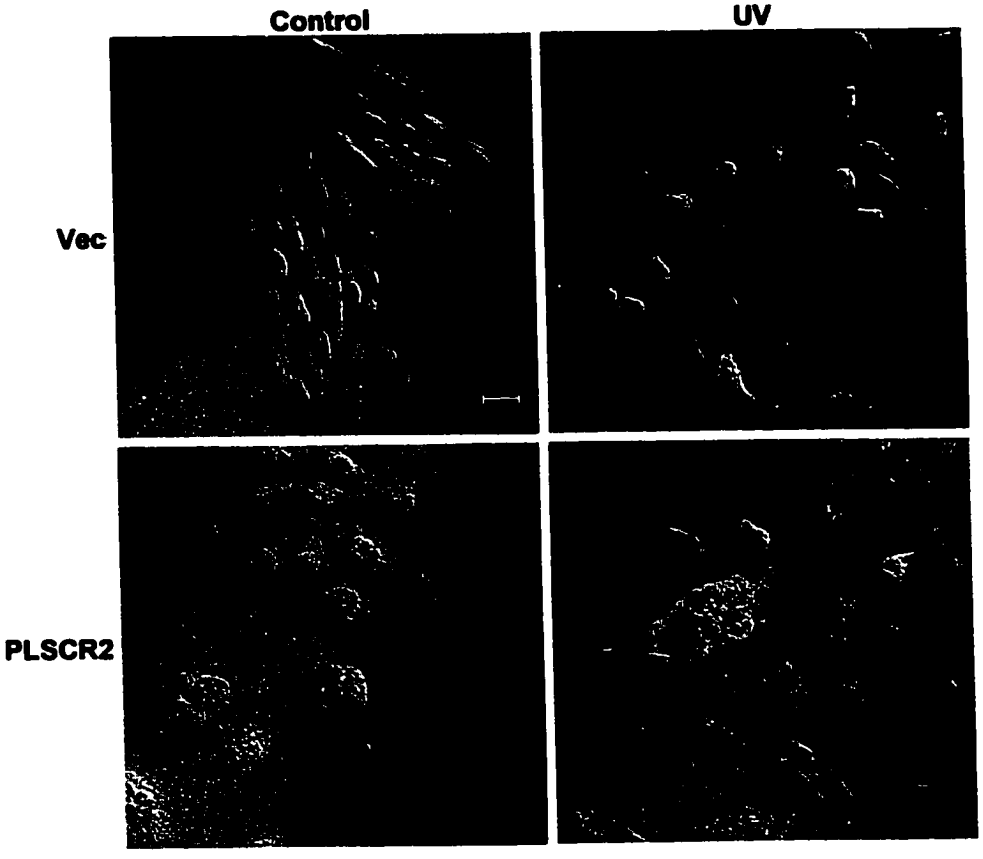

Figure 34. PtdSer externalization following UV irradiation in CHO-K1 cells over-expressing PLSCR2. CHO-K1 cells over-expressing PLSCR2 and control clones were treated without or with UV light; cells were further cultured for 6 h. FITC-annexin-V (green) and propidium iodide (red) binding assays were performed. Bar, 20 μm.

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 over-expressing PLSCR1 and PLSCR2. After UV-irradiation of cells and a 12-h incubation with [^{3}H]serine, there was a 2-fold increase in PtdSer synthesis in control cells compared to those with no UV-treatment (Fig. 35; top panel). PLSCR2 cells had a similar basal and UV-stimulated rate of PtdSer biosynthesis compared to control cells. Without UV irradiation, the basal rate of PtdSer synthesis was slightly higher (1.7-fold) in two PLSCR1 clones. With UV-stimulation there was a significant increase in UV-induced PtdSer biosynthesis in PLSCR1 cells; in two clones tested, PtdSer biosynthesis was 1.8-fold and 3.0-fold higher compared to the increase in UV-treated control cells. While PtdSer decarboxylation to PtdEtn slightly decreased following UV irradiation in control and PLSCR2 cells, decarboxylation was not altered in one PLSCR1 clone, and was increased slightly in the other (Fig. 35; middle panel). SM biosynthesis was stimulated (1.5 to 2.5-fold) in all cells following UV irradiation (Fig. 35; bottom panel). A higher amount of newly synthesized PtdSer also was recovered from the medium (representing mainly vesicles released during apoptosis) with UV-treated PLSCR1 cells compared to controls (Fig. 36). *De novo* biosynthesis of PtdCho 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 in control cells by 6% and unchanged in PLSCR1 cells with UV treatment (Table 4). Thus, over-expression 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.

Figure 35. Biosynthesis of serine-derived phospholipids in CHO-K1 cells over-expressing PLSCR1 and PLSCR2. CHO-K1 cells over-expressing PLSCR1 (A and B represent 2 clones) or PLSCR2 and control clones were grown in serine-free DMEM. Following treatment without (white bar) or with (black bar) UV light for 10 min, [^{3}H]serine (20 μCi) was added to cells. Cells were incubated for 12 h and harvested and lipids were extracted and separated by TLC. Radioactivity in PtdSer, PtdEtn, and SM was determined. Data are mean ± SEM of 6 samples.

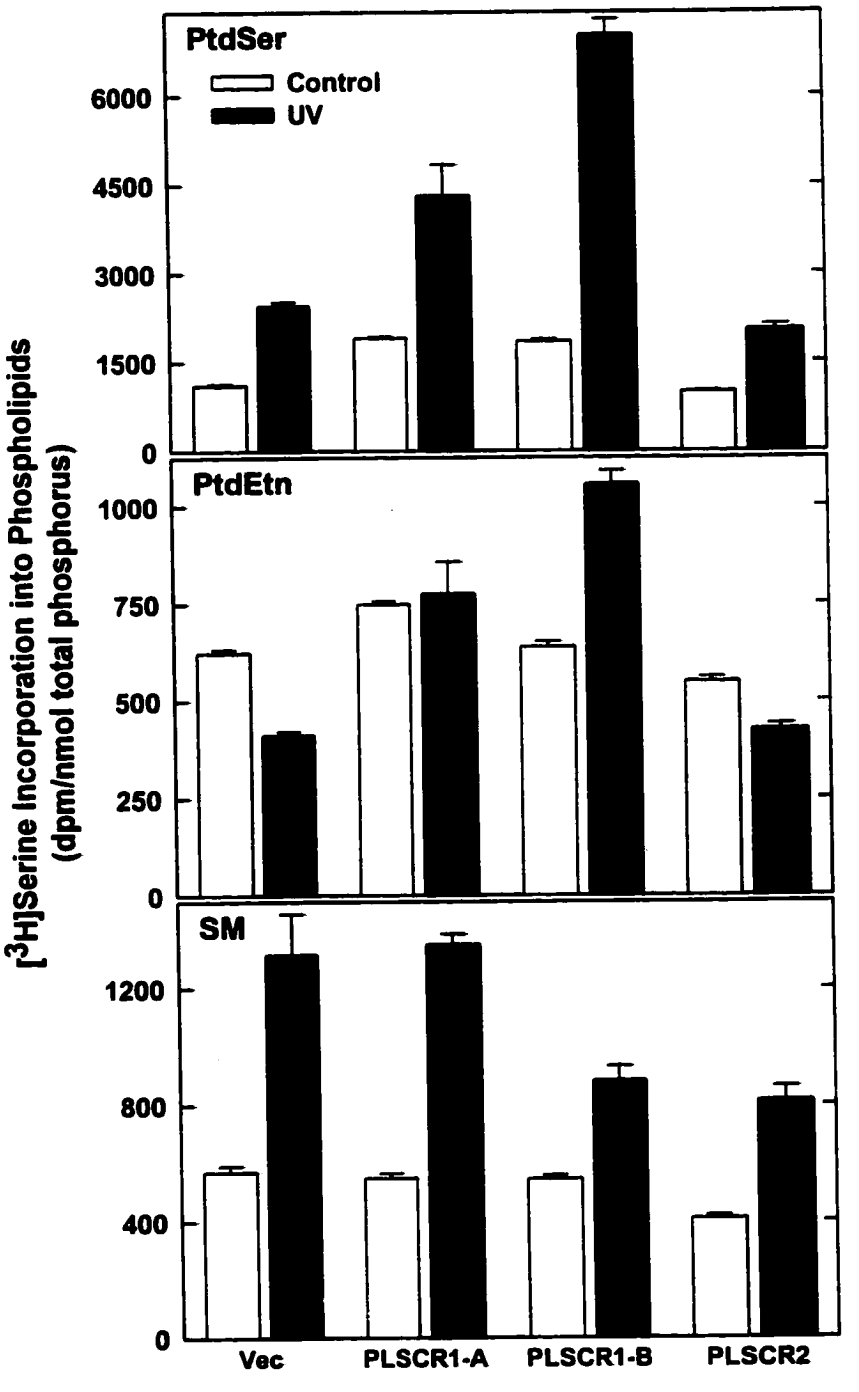
PtdSer
Control
UV
6000
4500
3000
1500
0
PtdEtn
1000
750
500
250
0
SM
1200
800
400
0
[3H]Serine Incorporation into Phospholipids (dpm/nmol total phosphorus)
Vec
PLSCR1-A
PLSCR1-B
PLSCR2

Figure 35

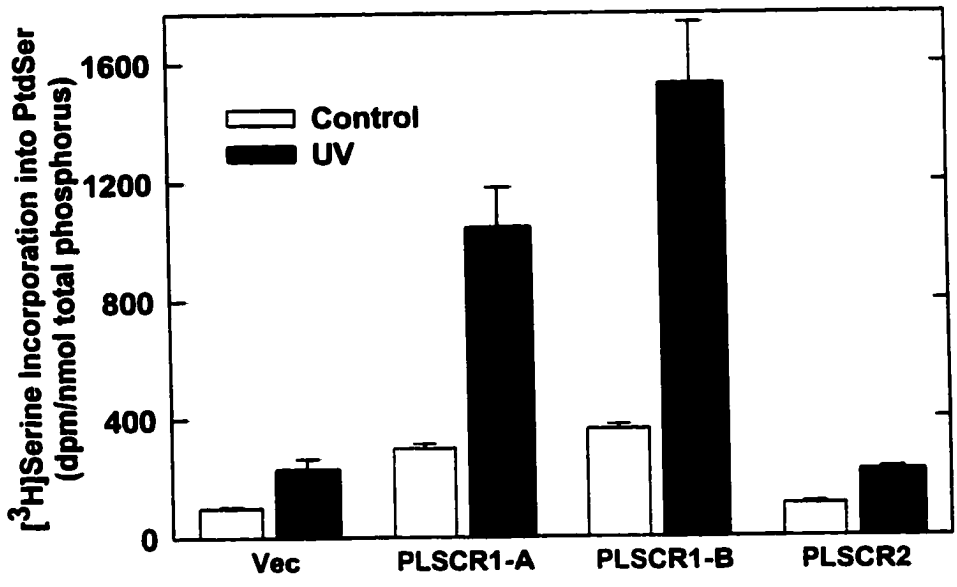

Figure 36. Recovery of PtdSer in culture medium following UV irradiation. Cell treatment and incubation were as described for Figure 35. After incubation, medium was collected and lipids were extracted and separated by TLC. Radioactivity in PtdSer from medium was determined. Data are mean ± SEM of 6 samples.

Table 4. PtdCho and PtdEtn biosynthesis in cells over-expressing PLSCR1 through CDP-choline and CDP-ethanolamine pathways

	PtdCho	PtdEtn
	[^{3}H]choline incorporation (1×10^4 dpm/nmol phosphorus)	[^{14}C]ethanolamine incorporation (1×10^3 dpm/nmol phosphorus)
Vector cells		
Control	1.98 ± 0.03	1.30 ± 0.01
UV	1.79 ± 0.08	1.09 ± 0.01*
PLSCR1 cells		
Control	2.66 ± 0.06	0.75 ± 0.05
UV	1.99 ± 0.04*	0.87 ± 0.02

* $p < 0.0001$ versus control

CHO-K1 cells over-expressing PLSCR1and control clones were grown in choline-free DMEM. Following treatment without or with UV irradiation for 10 min, [^{3}H]choline or [^{14}C]ethanolamine (3 μCi) was added to cells. Cells were incubated for 12 h and harvested as described in Methods; lipids were extracted and separated by TLC. Radioactivity in PtdCho or PtdEtn was determined. Data are mean ± SEM of 3 samples. The level of significance was assessed with a Student's *t*-test for unpaired data.

5. Caspase dependency 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 (Fig. 20). When 100 μM z-VAD-fmk was added to cells after UV irradiation, apoptosis was delayed in both control and PLSCR1 cells, based on morphology changes at different stages of apoptosis (Fig. 37). The basal level of cell death in cells over-expressing PLSCR1 was higher with significant PARP cleavage detected under normal conditions (Fig. 38C). In control cells, PARP cleavage, a marker for caspase-3 activation, was completely blocked by 100 μM z-VAD-fmk when apoptosis was induced by UV irradiation (Fig. 38C). z-VAD-fmk inhibited PARP cleavage in both control and UV-treated PLSCR1 cells. PtdSer externalization was also greatly inhibited by the presence of z-VAD-fmk both in UV-irradiated PLSCR1 and control cells (Fig. 38; A and B). Stimulation of PtdSer biosynthesis was unchanged in the presence of z-VAD-fmk in control cells. Further stimulation of PtdSer biosynthesis as a result of PLSCR1 over-expression was blocked by z-VAD-fmk (Fig. 39A). Movement of newly synthesized PtdSer into microvesicles was sensitive to z-VAD-fmk in both control and PLSCR1 cells (Fig. 39B). Inhibition of PtdSer decarboxylation and stimulation of SM synthesis were reversed by z-VAD-fmk in PLSCR1 cells but were not effected in control cells (Fig. 40). Thus, stimulation of PtdSer biosynthesis in PLSCR1 cells was dependent on caspase activation, while caspase-independent mechanisms were involved in up-regulating PtdSer biosynthesis in control cells after induction of apoptosis by UV exposure.

Figure 37. Prevention of UV-induced apoptosis in CHO-K1 cells by caspase inhibitor. CHO-K1 cells expressing PLSCR1 and control clones were treated without or with UV light; z-VAD-fmk (100 μM) was added to cells as indicated. After 12 h of incubation, phase contrast photographs were taken at 300X magnification.

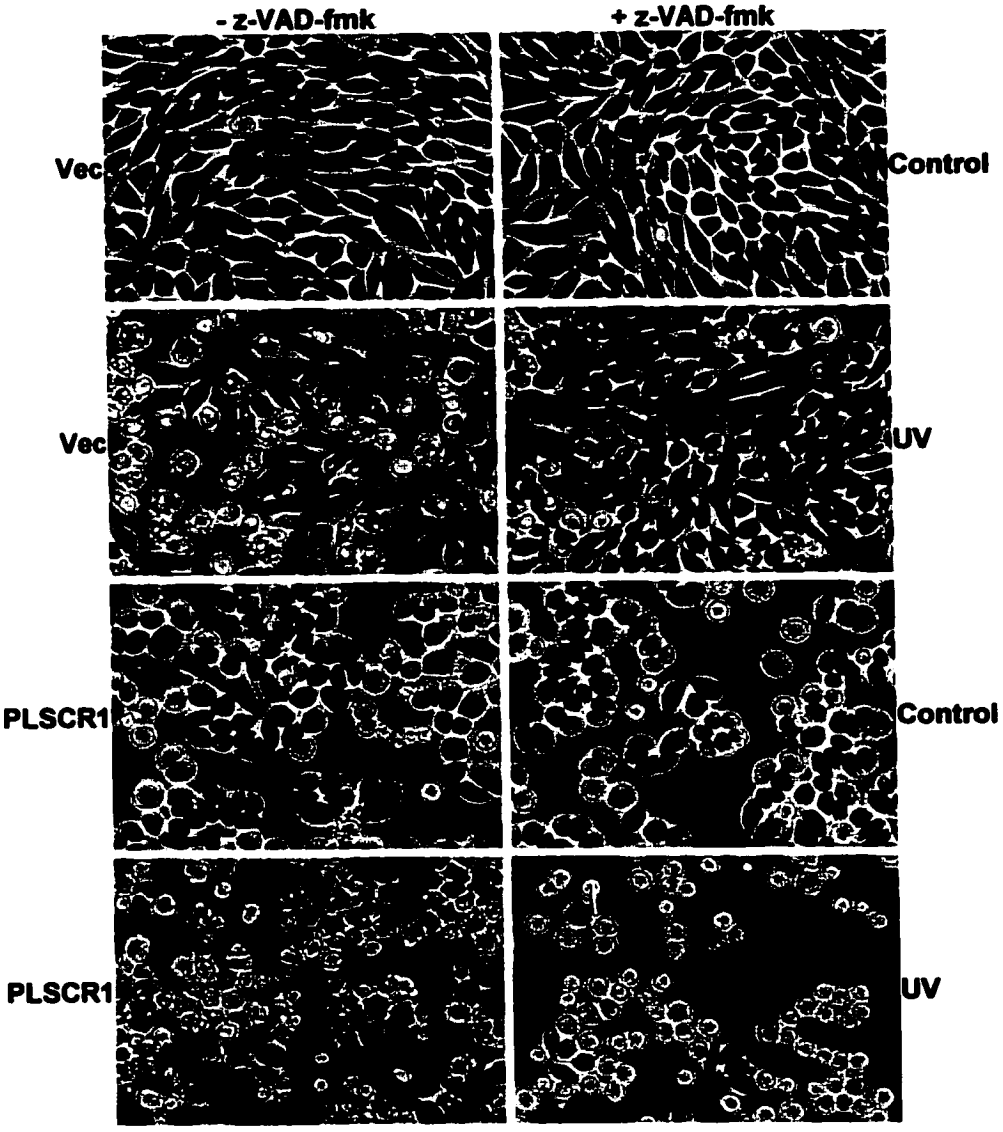
- z-VAD-fmk
+ z-VAD-fmk
Vec
Control
Vec
UV
PLSCR1
Control
PLSCR1
UV

Figure 37

Figure 38. Inhibition of UV-induced PtdSer externalization and PARP cleavage by caspase inhibitor. CHO-K1 cells over-expressing PLSCR1 and control clones were treated without or with UV light; z-VAD-fmk (100 μM) was added to cells as indicated. Panels A and B, after 8 h of incubation, cell surface exposure of PtdSer was assayed with annexin-V-FITC (green) and propidium iodide (red) staining. Bar, 20 μm. Approximately 500 cells were counted for positive staining with annexin-V in each sample. Values are mean ± SEM for 3 separate experiments. Panel C, cells were harvested 12 h after UV irradiation and proteins were extracted and separated (20 μg) using SDS-PAGE. Immunoblotting was performed with anti-human PARP pAb.

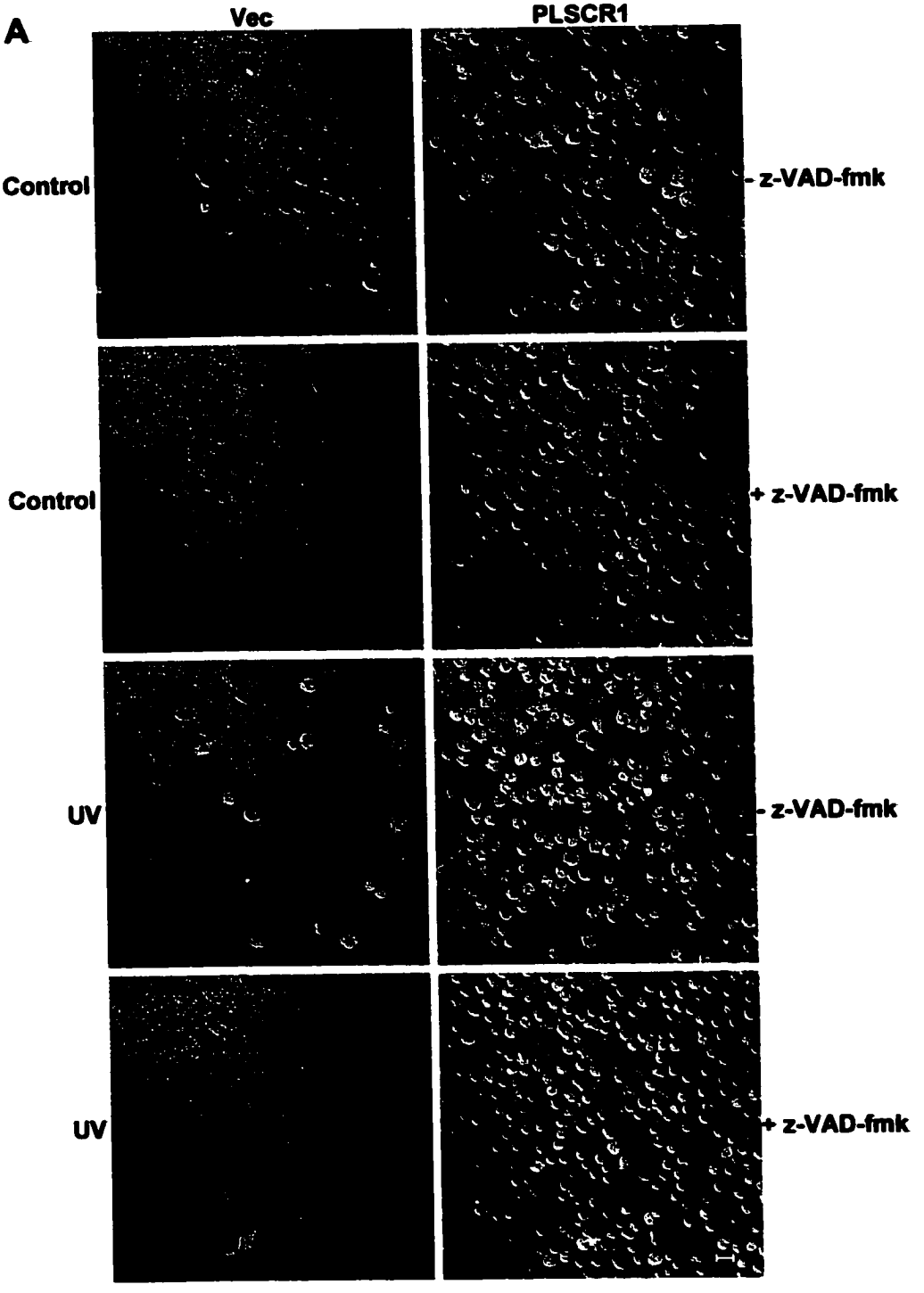
A
Vec
PLSCR1
Control
- z-VAD-fmk
Control
+ z-VAD-fmk
UV
- z-VAD-fmk
UV
+ z-VAD-fmk

Figure 38

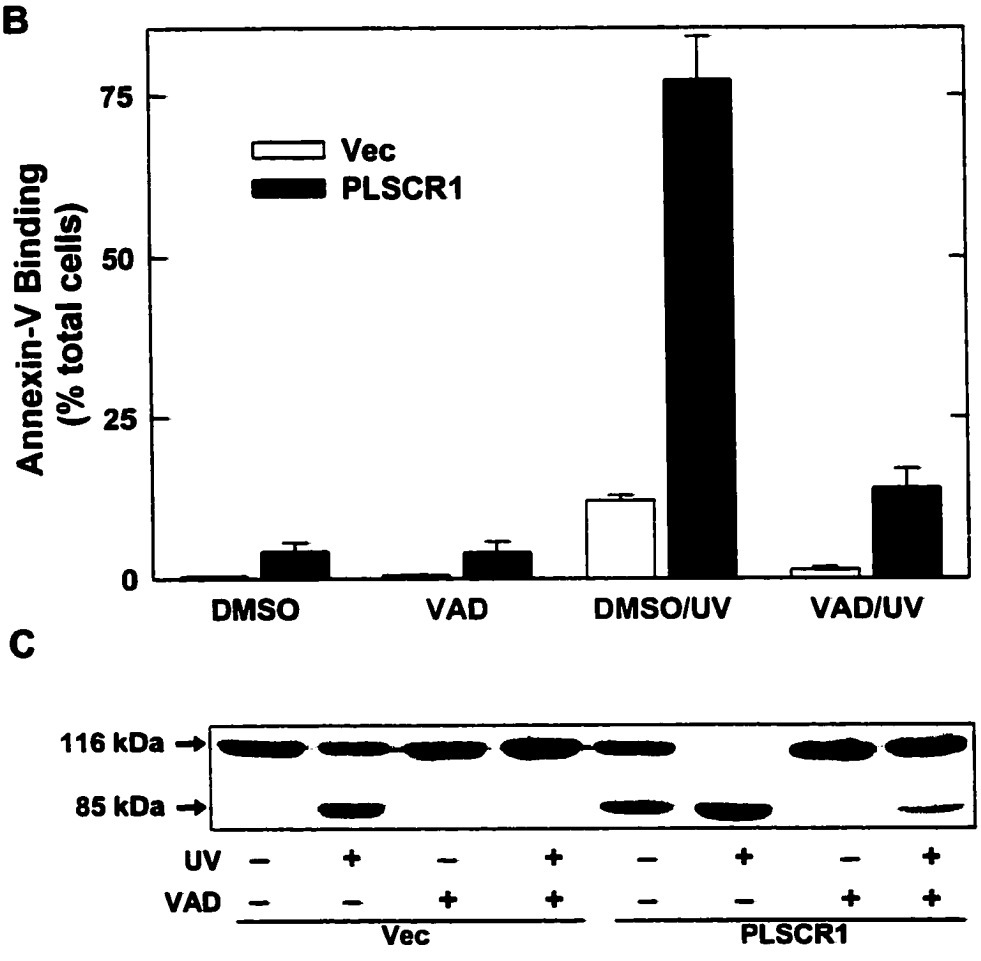
B
75
50
25
0
Annexin-V Binding
(% total cells)
Vec
PLSCR1
DMSO
VAD
DMSO/UV
VAD/UV
C
116 kDa
85 kDa
UV
VAD
Vec
PLSCR1

Figure 38

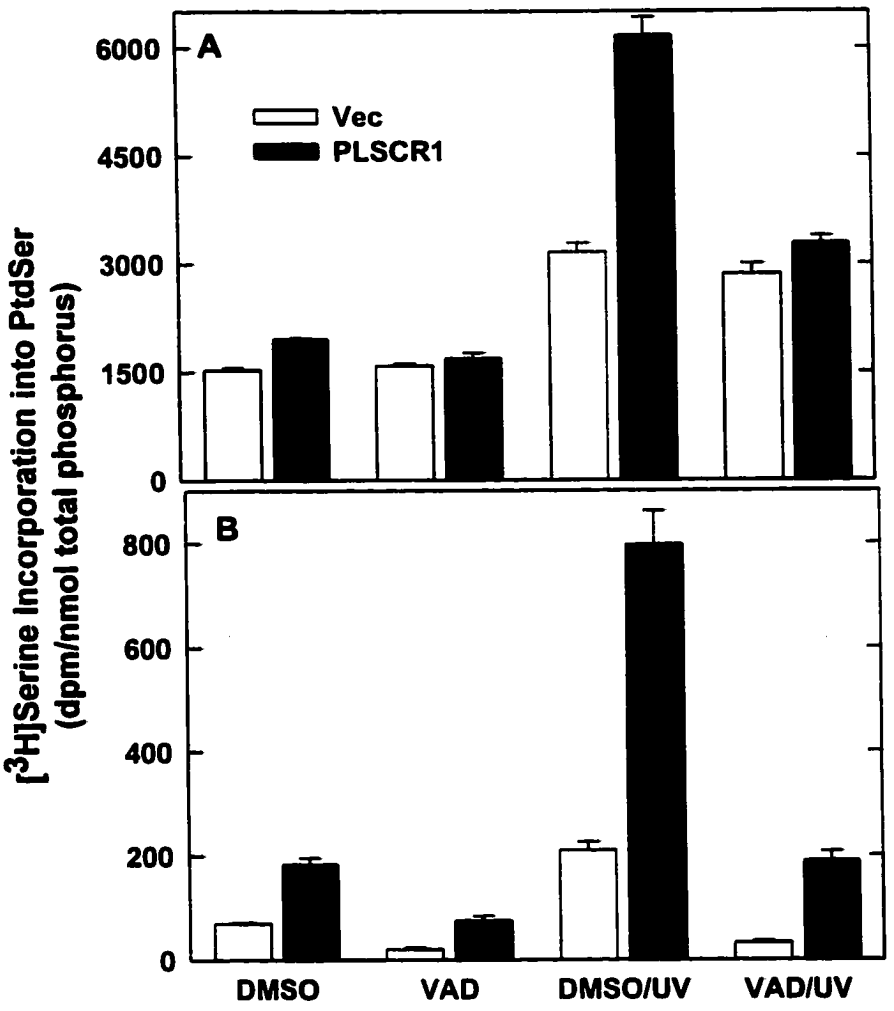

Figure 39. Effects of caspase inhibitor on PtdSer synthesis in CHO-K1 cells over-expressing PLSCR1 following UV irradiation. PLSCR1-expressing clones and control cells were grown in serine-free DMEM. Following treatment of cells without or with UV light, z-VAD-fmk (100 μM) was added to cells as indicated. Cells were incubated for 12 h in the presence of 20 μCi [^{3}H]serine. Cells were harvested and lipids were extracted and separated by TLC. Radioactivity in PtdSer was determined. Values are mean ± SEM of 6 samples. Panel A, total PtdSer from cell and medium; panel B, PtdSer from medium.

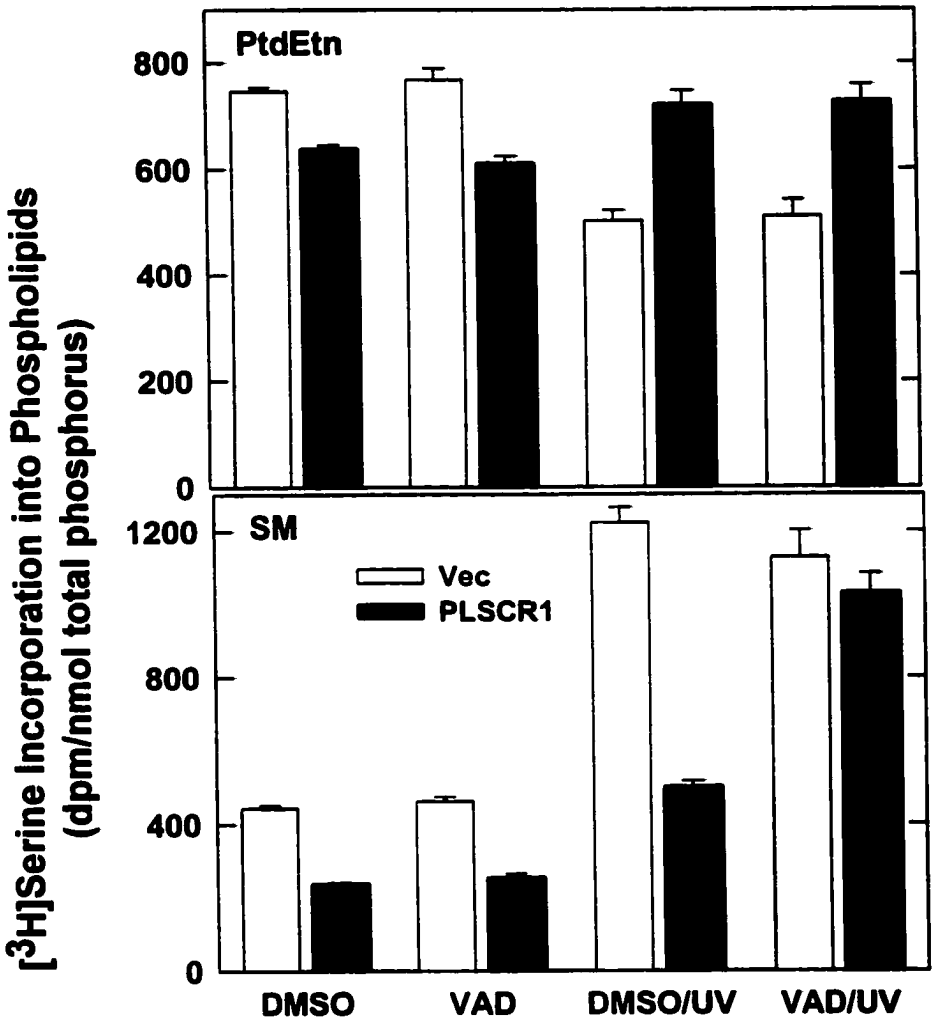

Figure 40. Effects of caspase inhibitor on PtdEtn and SM synthesis in CHO-K1 cells over-expressing PLSCR1 following UV irradiation. PLSCR1 and control cells were treated as in Figure 39. Radioactivity in PtdEtn and SM was determined. Values are mean ± SEM of 6 samples.

V. Discussion

A. Stimulation of PtdSer biosynthesis during programmed cell death.

The series of studies outlined in this thesis, using primarily two cell lines and several approaches to inducing apoptosis, were designed to determine whether PtdSer that appears on the outer surface of cells during apoptosis is newly synthesized and how general phospholipid synthesis relates to the appearance of other phospholipids in budding apoptotic bodies.

1. Caspase-dependent stimulation of PtdSer formation in U937 cells

Our initial data using U937 cells indicated that during CAM-induced apoptosis, PtdSer biosynthesis increased in a time-dependent manner that was proportional to the concentration of CAM. Synthesis of PtdSer was stimulated to a greater extent than that of the other major membrane phospholipids. Appreciably lower increases in SM and PtdEtn synthesis and a minor decrease in PtdCho synthesis were observed in total cells. Further, newly synthesized PtdSer was moved rapidly to the plasma membrane and was preferentially associated with membranes in apoptotic bodies. When apoptosis was initiated, PtdSer was transferred at levels 8-fold greater than in unstimulated controls whereas SM or PtdCho transfer to apoptotic bodies was only 2-3 fold compared to controls, although PtdCho was the most extensively transferred phospholipid in terms of total mass or radioactivity. The substantially greater relative transfer of PtdSer is significant because compared to SM and PtdCho, PtdSer is a minor plasma membrane component (Colbeau et al., 1971). As only cells in the G1 and S phase of the cell cycle are sensitive to CAM-induced cell death, the extent of enhancement was limited in unsynchronized cells because a maximum of 50% of the cells die as a result of CAM treatment. To circumvent the mixed signals obtained with cell cultures at different stages

of the cell cycle, cell division was synchronized by modifying the culture medium so that a greater portion of the population would be susceptible to induced apoptosis.

Apoptosis also was induced through activation of TNF receptors or by UV-mediated DNA damage in U937 cells. These treatments effectively induced maximal programmed cell death (>95%) and gave greater enhancement of PtdSer formation. The increase under these conditions was specific for PtdSer as levels of the other major phospholipids remained unchanged. Different mechanisms of stimulation of PtdSer synthesis may be involved. The TNF family mediates apoptosis through caspase-8 activation whereas DNA damage results in mitochondrial release of cytochrome C and subsequent activation of caspase-9 through formation of apoptosomes (Strasser et al., 2000). Thus, specific up-regulation of PtdSer synthesis that occurs rapidly during apoptosis may be a general biochemical change in response to two distinct apoptotic pathways. Depending on the relative effectiveness of the stimulation of cell death by different initiators, differential enhancement of *de novo* PtdSer synthesis is achieved.

The three methods of inducing apoptosis that we investigated all indicated that externalization of PtdSer is a downstream event of caspase activation (Vanags et al., 1996; Martin et al., 1996; Naito et al., 1997). We hypothesized that stimulation of PtdSer biosynthesis during apoptosis is directly related to PtdSer externalization and that changes in PtdSer biosynthesis may be controlled through caspase activation. Our data indicate that both increased PtdSer synthesis and accumulation of newly synthesized PtdSer in apoptotic bodies were abrogated when caspase activation was blocked by z-VAD-fmk, a broad-spectrum caspase inhibitor shown to inhibit PtdSer externalization and formation of microvesicles (Naito et al., 1997). Thus, enhanced formation,

externalization and transfer of newly synthesized PtdSer to apoptotic bodies in U937 cells appear to be directly related to caspase-mediated pathways.

Interestingly, other studies suggest that although z-VAD-fmk can inhibit caspase-mediated apoptosis induced by TNF-α/CHX in U937 cells, it also leads to rapid necrosis mediated by excessive formation of reactive oxidative species (Khwaja and Tatton, 1999). We observed a similar phenomenon, as intact cells were lost after simultaneous incubation of U937 cells with TNF-α/CHX and z-VAD-fmk. The fact that z-VAD-fmk effectively blocked the stimulation of PtdSer synthesis in this case indicates that regulation of PtdSer synthesis is a caspase-mediated process and is not related to necrosis. In some cells, PtdSer externalization occurs independent of caspase activation during apoptosis (Weil et al., 1998); whether PtdSer synthesis is increased in these models is not known.

Detailed knowledge of the regulation of *de novo* biosynthesis of PtdSer is limited. Turnover of PtdSer in cells is tightly regulated so that normally a constant level of PtdSer is maintained, possibly through PtdSer feedback control or other unknown mechanisms (Nishijima et al., 1986). Exposure of PtdSer to the cell surface and subsequent shedding of apoptotic bodies may signal a decrease in intracellular levels of PtdSer and thus trigger an increase in *de novo* synthesis of PtdSer. It is also possible that the release of PtdSer inhibition through transport of PtdSer to the cell surface and subsequent cellular budding maybe be influenced by caspase-mediated proteolysis. Our data indicate that activation of PtdSer synthesis requires caspase activity during apoptosis. No direct relationships between PSS I or II activity and activation of members of the caspase family had been previously demonstrated.

In conclusion, our studies in U937 cells showed that stimulation of PtdSer biosynthesis and movement of PtdSer to apoptotic bodies during apoptosis are important biochemical changes that are regulated by caspase pathways. These observations of a specific effect on PtdSer relative to other major membrane phospholipids suggest that altered PtdSer synthesis may influence the programmed cell death process and that regulation of PtdSer synthesis could be a point of altering the progression of apoptosis.

2. Caspase-independent stimulation of PtdSer formation in CHO-K1 cells

To see the extent to which the stimulation of PtdSer synthesis and externalization might be generally found in all cells during apoptosis, we further investigated PtdSer biosynthesis during UV-induced apoptosis in CHO-K1 cells. Genetic defects in CHO-K1 cells block cell-cycle arrest and thus favor initiation of apoptosis in response to UV-induced DNA damage (Tzang et al., 1999a; Tzang et al., 1999b). Our studies demonstrated that PtdSer externalization and other apoptotic changes developed in CHO-K1 cells as a result of UV irradiation. Furthermore, PtdSer biosynthesis also was enhanced as was the exposure of PtdSer. This enhanced activity and transfer seems to result from direct stimulation of serine base-exchange reactions instead of the blockage of PtdSer decarboxylation or increased serine uptake. Thus, the stimulation of PtdSer formation during apoptosis in both U937 and CHO-K1 cells suggest that altered PtdSer metabolism alone may not be a cell-type specific response, but rather may accompany PtdSer externalization in many types of apoptotic cells.

In contrast to observations with U937 cells, up-regulation of PtdSer biosynthesis in apoptotic CHO-K1 cells seemed to be less specific for PtdSer as SM formation was enhanced at levels similar to that of PtdSer. Newly synthesized SM also was transported

to apoptotic bodies so that an enrichment relative to whole cells was similar to that for PtdSer. The stimulation of both PtdSer and SM biosynthesis were insensitive to z-VAD-fmk in CHO-K1 cells indicating a caspase-independent regulation of phospholipid biosynthesis in these cells. Thus, our data indicate that increased PtdSer biosynthesis may be a general phenomenon during apoptosis but stimulation of PtdSer synthesis may involve distinct regulatory pathways depending on the types of cells involved.

Our studies focused on potential correlations underlying translocation of PtdSer at the plasma membrane and *de novo* biosynthesis of this phospholipid. We considered the possibility that PtdSer externalization may influence biosynthesis of new PtdSer because of disturbances in the intracellular distribution of PtdSer. Caspase-dependent or independent modulation of PtdSer synthesis during apoptosis may serve as a model for future study of the metabolic regulation of PtdSer biosynthesis and interorganelle trafficking. Any one of the steps involved could be potential targets for alteration of apoptotic processes. To advance this hypothesis, we moved to more complex models involving the over-expression of key enzymes catalyzing *de novo* PtdSer biosynthesis and externalization in CHO-K1 cells.

B. Involvement of PtdSer synthases in regulating stimulation of PtdSer biosynthesis

PtdSer formation in mammalian cells is catalyzed by at least two PtdSer synthase isoforms. PSS I utilizes PtdCho as the acceptor for serine in a base-exchange reaction to form PtdSer, whereas PtdEtn is the preferred acceptor substrate for PSS II (Kuge et al., 1997; Saito et al., 1998). Stimulation of PtdSer biosynthesis observed during apoptosis could involve the regulation of either PSS I or PSS II activities. PSS I enzyme is directly

inhibited by PtdSer so that over-expression of PSS I may not necessarily lead to increased PtdSer biosynthesis and change of PtdSer mass *in vivo* (Kuge et al., 1998). In contrast, PSS II enzyme is not sensitive to PtdSer by itself but apparently is subject to PtdSer feedback inhibition through a putative "PtdSer sensor". This latter intermediate molecule apparently responds to intracellular levels of PtdSer and mediates inhibition of PSS II activity through unknown mechanisms. When PSS II protein is over-expressed, feedback inhibition of PSS II is limited by overall availability of this PtdSer-sensing intermediate. Consequently not all PSS II activity is inhibited leading to higher levels of PtdSer synthesis when PSS II is over-expressed (Kuge et al., 1999). In our studies, cells over-expressing PSS I showed a slightly increased basal level of serine incorporation into PtdSer, whereas PSS II over-expression resulted in a 1.5-fold increase in PtdSer formation and a 2-fold increase in PtdSer decarboxylation. These observations support the possibilities of a direct effect on PSS I activity and an indirect effect on PSS II activity through PtdSer-mediated feedback inhibition.

When PtdSer is externalized to the cell surface and subsequently removed by shedding of PtdSer-containing microvesicles, cells may be able to sense decreased intracellular levels of PtdSer and activate PtdSer biosynthesis through activities of serine base-exchange enzymes. We propose that if PSS I and PSS II are directly involved, even greater stimulation of PtdSer formation can result from over-expression of these enzymes in CHO-K1 cells undergoing UV-induced apoptosis. Indeed, we found that CHO-K1 cells over-expressing either PSS I or PSS II showed much higher levels of PtdSer biosynthesis in response to UV irradiation indicating involvement of these enzymes in

enhancing PtdSer biosynthesis. In contrast, relatively little change was observed in the metabolism of other major phospholipids derived from serine.

We also postulated that increased *de novo* PtdSer biosynthesis due to over-expression of PSS I and PSS II may positively regulate PtdSer externalization and as a result influence the progression of UV-induced apoptosis. Surprisingly, UV-induced stimulation of serine incorporation into PtdSer in cells over-expressing PSS I or PSS II did not result in an increase in PtdSer externalization (no major differences were observed in externalization of PtdSer in cells expressing PSS isoforms or in their controls). Furthermore, apoptosis was negatively, instead of positively, regulated by over-expression of PSS I or II in UV-irradiated cells, proven by a lack of caspase-3 activation and other apoptotic morphological changes. An increase in PtdSer biosynthesis in UV-treated PSS-expressing cells occurred shortly after UV irradiation (3-4 h) and thus was independent of, or preceded, PtdSer externalization (8 h) as apoptosis was prevented or delayed in these cells. In contrast to caspase-dependent PtdSer stimulation in U937 cells, activation of caspases seems to be uncoupled from PtdSer biosynthesis in CHO-K1 cells and also is not required for regulating the activity of either PSS I or PSS II. PtdSer biosynthesis seems to be catalyzed by PSS I or PSS II as a caspase-independent event. The increase of PtdSer biosynthesis in cells over-expressing PSS I and PSS II precedes externalization of PtdSer indicating that their activities may be regulated by events upstream of apoptosis rather than associated with PtdSer externalization. These observations indicate limitations to the possibility of a universal causal relationship between PtdSer externalization and the up-regulation of PtdSer biosynthesis.

The greater stimulation of PtdSer biosynthesis in PSS-expressing cells following UV irradiation appears to be a direct result of high levels of expression of these serine base-exchange enzymes. By extrapolation (but not proven in our studies), PSS I and/or PSS II may be up-regulated in wild type CHO-K1 cells following UV exposure. PSS I and/or PSS II catalyze the incorporation of serine into PtdSer in response to UV irradiation in a caspase-independent manner. The mechanisms of stimulation of serine base-exchange enzymes have yet to be identified. Activities of serine base-exchange enzymes are stimulated by Ca^{2+} (Hübscher, 1962) and depletion of Ca^{2+} from ER stores strongly inhibits PtdSer biosynthesis (Pelassy et al., 1992a). Changes in intracellular levels of Ca^{2+} are common early responses in many forms of induced apoptosis (McConkey and Orrenius, 1996a; McConkey and Orrenius, 1996b). Thus, increased activities of PSS I and PSS II may be modulated through calcium-mediated processes but these appear to be independent of caspase activity. Serine base-exchange reactions can be stimulated by cationic amphiphilic chemicals, K^{+}-channel blockers and calmodulin antagonists (Pelassy et al., 2000a). The involvement of these stimulators of PtdSer biosynthesis during induced apoptosis also may contribute to the increase in new PtdSer formation.

Lack of PtdSer externalization and apoptosis in PSS-expressing cells treated with UV light was unexpected. The reasons for this resistance to enhanced apoptosis in PSS I or PSS II expressing cells remain unknown but seem to be specific for UV-induced apoptosis. These over-expressing cells showed responses similar to their control counterparts when treated with other death stimuli such as staurosporine or farnesol. UV irradiation causes cell cycle arrest and DNA damage. Failure to correct the latter results in the cell death due to activation of caspase dependent pathways mediated by p53

(Kulms and Schwarz, 2000). Thus, it seems likely that upstream events normally associated with UV-induced apoptosis may be inhibited when PSS I or PSS II are over-expressed. Whether this is directly caused by an excessive amount of PSS I or PSS II enzymes *per se* or is a result of higher levels of intracellular PtdSer requires further assessment. PtdSer serves as a co-factor for proteins crucial in cell signaling, such as PKC and MARCKS (Rando, 1988; Taniguchi and Manenti, 1993). It can be speculated that increased intracellular levels of PtdSer may result in the recruitment of PtdSer-associated proteins and thus influence the progress of apoptosis. Possibly high levels of PtdSer inside the cell may serve as a survival signal. Accordingly, the process of translocation of PtdSer to the cell surface may have positive effects on the progression of apoptosis. Although we speculate that exposure of PtdSer on the cell surface may provide a signal for enhancement of PtdSer biosynthesis, it appears that an increase in intracellular PtdSer levels in response to UV irradiation in conjunction with over-expressing PSS I or PSS II did not elicit the same level of regulation or positive feedback on PtdSer translocation.

In conclusion, the data from this series of experiments show that over-expression of PSS I or PSS II specifically enhances stimulation of PtdSer formation after UV irradiation, indicating that increasing the content of synthase enzymes can up-regulate PtdSer biosynthesis. However, PtdSer formation through PSS I and PSS II activity is uncoupled from caspase activation. Moreover, cells over-expressing PSS I or PSS II showed significant resistance to UV-induced apoptosis. These studies provide a better understanding of the role or limitations of PtdSer biosynthesis and movement in the process of apoptosis. Furthermore, regulation of PtdSer biosynthesis and its

externalization may allow for consideration of novel ways of altering induction or inhibition of apoptosis.

C. Activation of PtdSer externalization during apoptosis regulates PtdSer biosynthesis

Studies of cells over-expressing PtdSer synthases did not provide evidence for a direct relationship between PtdSer externalization and PtdSer biosynthesis. Stimulation of PtdSer biosynthesis preceded PtdSer externalization but higher intracellular levels of PtdSer failed to increase PtdSer externalization in cells over-expressing PSS I or PSS II. To test the postulated relationship between PtdSer externalization and its biosynthesis from another approach, we studied the involvement of PLSCR isoforms, the key enzymes responsible for actively translocating PtdSer to the cell surface, in regulating PtdSer biosynthetic pathways. Stable over-expression of two PLSCR isoforms was established in CHO-K1 cell lines. As endogenous PLSCR1 cannot be detected in CHO-K1 cells by Western blotting and no scramblase activity is detected by *in vitro* enzyme assays (Frasch et al., 2000), we anticipated that addition of this enzyme to the cells by transfection might enhance PtdSer synthesis and/or externalization in response to induction of apoptosis.

When over-expressed in CHO-K1 cells, PLSCR1 localized predominantly to the plasma membrane of the cell, and in some cases, to the nuclei. Palmitoylation at conserved cysteines in PLSCR1 is required for anchoring this protein to the plasma membrane (Zhao et al., 1998b); over-expressed PLSCR1 proteins not modified by palmitoylation may be moved into the nuclei. Changes in cell morphology occurred, basal cell death was higher and cells grew at a slower rate when PLSCR1 was over-expressed. Noticeably, PLSCR1-expressing cells also had higher basal levels of PtdSer expression on the cell surface. Significant membrane blebbing also was observed in these

cells. When PLSCR1 is stably expressed in ovarian carcinoma cell line HEY1B (Silverman et al., 2002), 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. Reduction in tumor cell size due to differentiation and infiltration of macrophages into tumors also occurs as a result of PLSCR1 expression (Silverman et al., 2002). Our data indicate that PLSCR1 can suppress cell growth and increase apoptosis in cell culture. The implications or mechanisms of morphological changes in PLSCR1 over-expressing cells are not clear, but increased membrane lipid movement and blebbing may contribute. PtdSer is suggested to be preferentially exposed in membrane domains, particularly in membrane blebs (Henson et al., 2001b), indicating that higher PLSCR1 activity may direct PtdSer to these surface regions and may explain the increased membrane vesiculation observed in PLSCR1 expressing cells.

When apoptosis was induced by UV irradiation, cells over-expressing PLSCR1 progressively developed morphological and biochemical changes indicative of apoptosis. First, PLSCR1 cells rapidly exposed PtdSer to the surface; by 8 h following UV-stimulation, the majority of the cells over-expressing PLSCR1 showed positive annexin-V binding whereas very few control cells had exposed PtdSer. Secondly, caspase 3 activation occurred much earlier in PLSCR1 cells compared to controls. Thirdly, 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 an anti-proliferative and pro-apoptotic enzyme, as its over-expression 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 radiolabeled serine as a precursor. Clones over-expressing PLSCR1 showed higher basal levels of PtdSer biosynthesis compared to vector control cells. When cells with PLSCR1 over-expression were treated with z-VAD-fmk, both PtdSer externalization and new PtdSer synthesis were reduced to basal levels of vector control cells. The basal levels of stimulation of PtdSer biosynthesis may be increased because more cells are expressing PtdSer on the surface of PLSCR1-expressing cells under control conditions. It is unlikely that activation of serine base-exchange enzymes contributes directly to this caspase-dependent stimulation in PLSCR1-expressing cells since PtdSer synthesis, catalyzed by over-expressed PSS I and PSS II, does not require caspase activation.

Stimulation of PtdSer biosynthesis was significantly higher in PLSCR1-expressing cells compared to control cells containing empty vector following UV irradiation and more newly synthesized PtdSer moved into microvesicles. Apoptotic cells from clone PLSCR1-A had a 1.5-fold stimulation of PtdSer synthesis while a 2.5-fold increase was achieved in clone PLSCR1-B compared to UV-treated control cells. Potentially, an increase in PtdSer level could be explained by a blockage of PtdSer decarboxylation but this apparently was not the case, as synthesis of serine-derived PtdEtn was not affected (PLSCR1-A) or was even slightly enhanced (PLSCR1-B) indicating increased transport of PtdSer into mitochondria. PLSCR1-A cells showed no changes in SM formation compared to treated control cells (2.5-fold stimulation following UV irradiation) whereas

PLSCR1-B had only 1.5 fold increase in SM formation. The discrepancy observed between the two clones of PLSCR1-expressing cells can be explained by variable expression rates of PLSCR1 proteins in these two clones; by immunofluorescence detection clone PLSCR1-A had a lower level of PLSCR1 expression than clone PLSCR1-B. It seems that when more PLSCR1 protein is present, PtdSer related biosynthetic and trafficking pathways are up-regulated to a greater extent whereas more down-regulation occurs in the SM biosynthesis pathway that shares a common substrate (serine). SM and PtdSer biosynthesis pathways may be regulated concertedly during apoptosis.

Similar to the parent CHO-K1 cells, vector control cells had a 2-fold stimulation of PtdSer synthesis following UV irradiation independent of caspase. The 6-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 cells back to that of the UV-treated control cells but did not reduce it to the level of untreated cells. We propose that UV-induced increases in PtdSer biosynthesis in CHO-K1 cells are mediated through serine base-exchange enzymes that function independent of caspase. PLSCR1, when over-expressed in cells, activates PtdSer externalization and consequently mediates stimulation of new PtdSer following UV irradiation. Since PtdSer externalization during apoptosis is under the control of caspase regulation during an activated death program, it is likely that stimulation of PtdSer formation resulting directly from PtdSer externalization becomes sensitive to caspase inhibition.

When PLSCR2, a shorter isoform of scramblase without an extracellular tail was expressed, the protein was found predominantly inside the nucleus and the cells were generally larger. When apoptosis was induced in cells expressing PLSCR2 by exposure

to UV light, no significant differences were observed in PtdSer externalization and progression of cell death was similar to control cells containing empty vector. Thus, PLSCR2 does not facilitate PtdSer externalization or alter PtdSer biosynthesis. 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 PLSCR1 (Zhou et al., 1998). Possibly, PLSCR2 was not targeted to the right region of our cells to influence PtdSer externalization and apoptosis, and its presence in the nucleus is inadequate to influence apoptosis. Accordingly, PM localization may be essential for PLSCR to regulate PtdSer redistribution in a way that promotes apoptosis.

In summary, our data from this series of experiments indicate that PLSCR1 is the scramblase involved in externalization of PtdSer at the plasma membrane. PLSCR2 isoform is targeted primarily to the nucleus where it is ineffective. Cells over-expressing PLSCR1 have increased and earlier PtdSer externalization and processes of apoptosis induced by UV irradiation also are facilitated. PLSCR1 may not be necessary for apoptosis but it can alter its progression. PtdSer biosynthesis is greatly stimulated in PLSCR1 over-expressing cells and this stimulation is dependent on caspase activation. 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.

D. Working model

Collectively, our data support a direct relationship between PtdSer externalization and biosynthesis of new PtdSer during apoptosis (Fig. 41). Stimulation of PtdSer biosynthesis in U937 cells is dependent on caspase activity. In CHO-K1 cells, UV-induced stimulation of PtdSer formation was independent of caspase activation, as it was not blocked by the caspase inhibitor, z-VAD-fmk. Positive regulation of the activities of

PSS I and PSS II may be involved in the stimulation of caspase-independent PtdSer synthesis following UV irradiation in wild type CHO-K1 cells and in cells over-expressing PSS I or PSS II enzymes. Mechanisms underlying this caspase-independent activation of serine base-exchange reactions remain unidentified although changes in intracellular or intra-compartmental Ca^{2+} level may play a regulatory role. Activation of PtdSer externalization to the cell surface, such as that seen through over-expression of PLSCR1, provides another signal to enhance PtdSer biosynthesis in both untreated and UV-treated PLSCR1 cells in a caspase-dependent manner. In this case, the caspase-regulated step appears to be PtdSer externalization mediated by PLSCR1. 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. 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.

U937 cells express significant amounts of endogenous PLSCR1 (Fadeel et al., 1999b) whereas in CHO-K1 cells, endogenous scramblase cannot be detected by Western blotting and activity assays (Frasch et al., 2000). In U937 cells, because of the existence of high levels of PLSCR1, caspase-dependent PtdSer externalization is likely to be responsible for triggering the increase in PtdSer biosynthesis during apoptosis. On the other hand, UV irradiation of CHO-K1 cells lacking PLSCR1 stimulates PSS I and PSS II activity in a caspase-independent manner. Additive effects are observed when both caspase-dependent and independent regulation of PtdSer biosynthesis coexist in CHO-K1 cells over-expressing PLSCR1.

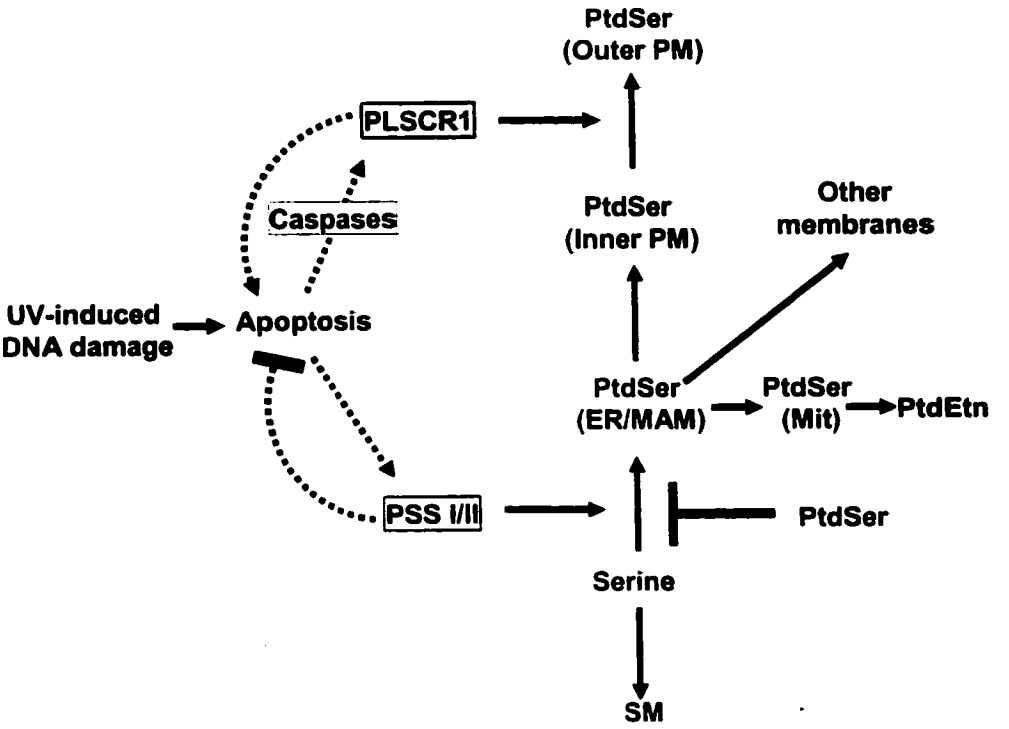

Figure 41. Proposed effects of PLSCR1 and PSS over-expression on PtdSer biosynthesis and apoptosis. In CHO-K1 cells, over-expression of PLSCR1 leads to enhanced PtdSer externalization to the cell surface. This releases the feedback control of PtdSer biosynthesis by the product itself. Consequently, *de novo* biosynthesis is stimulated. As PLSCR1-mediated PtdSer exposure requires the activation of caspases, stimulation of PtdSer biosynthesis during UV-induced apoptosis also is caspase-dependent. PLSCR1 over-expression positively regulates the progression of UV-induced apoptosis. On the other hand, over-expression of PSS I and II leads to enhanced PtdSer biosynthesis in response to UV irradiation in a caspase-independent manner. Over-expression of PSS I or PSS II negatively regulates the progression of UV-induced apoptosis.

Although several questions about the details of regulatory mechanisms remain unanswered, our studies contribute to a better understanding of the role of PtdSer biosynthesis and movement in the process of apoptosis. Stimulation of PtdSer formation following UV irradiation is maintained at high levels for a prolonged period, contradicting a previous report suggesting transient stimulation of PtdSer biosynthesis in apoptotic Jurkat cells (Aussel et al., 1998). Continued PtdSer synthesis may be required for sustained apoptosis. Whether restrictions to the supply of PtdSer will alter PtdSer externalization and the progression of apoptosis remains to be investigated. Furthermore, better understanding of the regulation of PtdSer biosynthesis and its externalization may allow for consideration of novel ways of altering the induction or progression of apoptosis. Over-expression of PLSCR1 positively influences the development of apoptosis through unknown mechanisms whereas over-expression of PSS I and PSS II negatively controls apoptosis induced specifically by UV irradiation. It is apparent from these studies that biosynthesis of PtdSer mediated by altered scramblase or PtdSer synthase activities is a potential point of regulation and hence a possible target for therapeutic intervention in the complex process of cell death or survival.

VI. Future directions

In this study, increased PtdSer biosynthesis and externalization of this phospholipid to the cell surface was observed in U937 and CHO-K1 cells induced to undergo apoptosis with various stimulators. We propose that PtdSer biosynthesis may be regulated through caspase-dependent or independent pathways depending on the cell type. The caspase-dependent stimulation of PtdSer formation seems to be associated with PLSCR1-mediated externalization of PtdSer while PtdSer synthases may be induced through caspase-independent pathways to up-regulate PtdSer biosynthesis. A recent report indicates that PtdSer biosynthesis is inhibited rather than stimulated in macrophages when induced to undergo apoptosis by group B streptococcus, (Buratta et al., 2002). Compared to U937 and CHO-K1 cells, macrophages constitutively express PtdSer at the cell surface and induction of apoptosis only increases the levels of PtdSer on the outer layer of the plasma membrane. Further expansion of our knowledge on this apoptosis-related event is necessary to determine whether stimulation of PtdSer biosynthesis is a general phenomenon during induced apoptosis, especially in cells with asymmetrical plasma membrane, and whether this event is directly related to PtdSer externalization or serine base-exchange enzymes. The use of PLSCR1-containing cells (HL-60 cells) or cells lacking endogenous PLSCR1, such as Raji cells (Fadeel et al., 1999b), in future experiments will help to confirm how central the new synthesis and externalization of PtdSer is to the apoptotic process.

U937 cells remain good models for study as they respond to death stimuli through all known major apoptotic signaling pathways. U937 cells grow in suspension and conventional transfection methods, such as lipofectamine transfection, are unable to

introduce plasmid DNA into these cells efficiently. In some of our exploratory experiments, we attempted to establish U937 cell lines stably over-expressing PSS or PLSCR enzymes using electroporation for transfection of plasmid DNA followed by dilution cloning. Clones were isolated but only very low levels of expression of foreign proteins were detected by biochemical analysis. Alternatively, these enzymes were over-expressed in CHO-K1 cells for further studies. It seems worthwhile to try novel transfection techniques that will lead to high expression levels of protein in U937 cells. Adenovirus infection techniques can reproducibly provide high levels of transient expression of foreign proteins in mammalian cells (Kovesdi et al., 1997). It could be extremely helpful to use these techniques in the study of proteins like PLSCR1 that may be pro-apoptotic and anti-proliferative but are difficult to express stably in cells.

We propose that stimulation of PtdSer biosynthesis in apoptotic U937 cells is triggered by PtdSer externalization controlled by caspase activation. It is unclear which caspase is involved but caspase-3 is unlikely as PtdSer externalization induced during TNF-α-mediated apoptosis in U937 cells can be uncoupled from caspase-3 activation by mitochondrial inhibitors, such as antimycin A and oligomycin (Zhuang et al., 1998). These reagents can be used to determine whether stimulation of PtdSer formation in U937 cells results directly from PtdSer externalization or from other caspase-3 related events. It also may help to determine whether an initiator caspase, such as caspase-9, is responsible for caspase dependency of PtdSer biosynthesis.

PtdSer in the inner layer of the plasma membrane can be subjected to oxidation mediated by cytochrome C release from mitochondria into the cytosol (Kagan et al., 2000). Oxidized PtdSer can be translocated outward to the cell surface and may directly

inhibit aminophospholipid translocase activity. This inhibition contributes to the induction of PtdSer externalization along with the activation of PLSCR activity during apoptosis. The fact that PtdSer externalization is related to potential changes of mitochondrial membranes seems to support an oxidative hypothesis for the modulation of PtdSer movement (Zhuang et al., 1998). Biochemical techniques, such as HPLC and mass spectrometry, can be utilized in future experiments to examine the extent to which newly synthesized PtdSer is oxidized in association with cytochrome C release and whether oxidized PtdSer is distributed to the cell surface.

CHO-K1 cells over-expressing PSS I or PSS II specifically resisted UV-induced apoptosis indicated by blockage of caspase-3 activation and subsequent apoptotic changes. UV irradiation induces DNA damage which initiates apoptosis through activation of p53; subsequent p53 signaling pathways stimulate transcription of key proteins involved in activating death programs mediated by death receptors or mitochondrial permeability transition. Mitochondrial changes release cytochrome C into the cytosol inducing formation of apoptosomes and activation of initiator caspases such as caspase-9 (Kulms and Schwarz, 2000). Future elaboration of the mechanisms underlying PSSI or II related-resistance to apoptosis could include assessment of upstream signaling events such as stabilization and activation of p53, expression of pro-apoptotic members of the Bcl-2 family, cytochrome C release or proteolysis of pro-caspase-9, in cells over-expressing PSS I or PSS II.

PtdSer is crucial for PKC activities (Nishizuka, 1992) and PKC isoforms contribute to the regulation of diverse cell responses such as cell proliferation and apoptosis (Dempsey et al., 2000). PtdSer-mediated regulation of PKC activity may result in

resistance to apoptosis and examination of PKC profiles in cells over-expressing PSS I or PSS II following UV irradiation may help to determine whether the PtdSer synthases *per se* or higher levels of PtdSer are responsible for the resistance.

Over-expression of PLSCR1 in CHO-K1 cells resulted in significant changes in the morphology of the epithelial cells making them smaller and more round in shape. Cytoskeleton organization of normal adherent cells is stabilized by asymmetric distribution of aminophospholipids (Manno et al., 2002). Changes of cell morphology in PLSCR1-expressing cells may result from the disturbance of lipid asymmetry or PLSCR1-mediated signaling pathways. Further examination of cytoskeletal reorganization in cells over-expressing PLSCR1 may provide insight into the involvement of PLSCR1 in regulating cell morphology or whether PtdSer externalization also plays a role.

UV-induced apoptosis in CHO-K1 cells is accelerated by over-expression of PLSCR1. One possible explanation is that increased PtdSer on the cell surface positively regulates the process of apoptosis. Another implicates PKC-δ in promoting cell death (Gschwendt, 1999). Preliminary data indicated that PLSCR1-expressing cells showed much higher levels of PKC-δ cleavage following UV irradiation compared to treated control cells. This cleavage, specific to apoptosis, may produce a constitutive active kinase fragment documented in many cell types as pro-apoptotic (Dempsey et al., 2000). Further studies could help to reveal whether the cleavage results from PLSCR1 signaling pathways or whether increased PKC-δ positively regulates PLSCR1 activity and progression of apoptosis. It also remains to be determined whether PKC-δ activity is involved in up-regulation of PtdSer biosynthesis in cells over-expressing PLSCR1, as

there is evidence that PKC-δ cleavage and activation are required in persistent PtdSer externalization during apoptosis (Frasch et al., 2000). This may provide important clues about potential causal relationships between PtdSer externalization and up-regulation of its biosynthesis.

PLSCR1 functions at the plasma membrane. It would be interesting to determine where apoptosis-specific stimulation of PtdSer biosynthesis occurs. ER and MAM membranes are considered to be the main sites of PtdSer formation (Stone and Vance, 2000). However, the possibility that PtdSer can also be formed rapidly on the plasma membrane (Xu et al., 1994; Vincent et al., 1999), possibly mediated by PLSCR1, cannot be ruled out. Cell fractionation followed by determination of enzyme activity and localization of newly synthesized PtdSer can be performed.

Stimulation of PtdSer biosynthesis appears to play important roles in regulating PtdSer externalization and progression of apoptosis. Inhibition of apoptosis-mediated PtdSer formation could be explored to assess effects on the exposure of PtdSer and apoptosis. Exposure of cells to exogenous PtdSer could be one way to turn off endogenous biosynthesis (Nishijima et al., 1986). Experiments with CHO-K1 cells cultured in medium supplemented with exogenous PtdSer could be used to examine effects on PtdSer externalization and biosynthesis as well as the progression of cell death in response to UV irradiation. Other chemical inhibitors of PtdSer biosynthesis may also be tested (Pelassy et al., 2001; Pelassy et al., 1989; Pelassy et al., 1991; Pelassy et al., 2000b). Such experiments might help to define the potential for use of PtdSer as an anti-aging and memory-improving supplement in medical practice as suggested in some reports (Crook et al., 1991; Amaducci et al., 1991).

In CHO-K1 cells, SM biosynthesis also was stimulated to an extent similar to that of PtdSer synthesis while changes in PtdCho and PtdEtn metabolisms were minor. SM has been suggested to be co-translocated during apoptosis along with PtdSer but in the opposite direction. This could lead to the hydrolysis of SM in the inner layer of the plasma membrane. Ceramide formed may be involved in the membrane blebbing process of apoptosis (Tepper et al., 2000). Whether increased and prolonged formation of SM during induced apoptosis in CHO-K1 cells participates in membrane vesiculation through such a hypothetical pathway is a future direction for investigation. It also would be interesting to determine why this stimulation of SM biosynthesis does not exist in apoptotic U937 cells.

Arg-95 of PSS I and Arg-98 of PSS II have been identified as essential for negative control of new PtdSer synthesis by exogenous PtdSer (Kuge et al., 1998; Kuge et al., 1999). Cell lines over-expressing PSS I or PSS II proteins with mutations at these key residues may provide new tools for gaining a better understanding of the stimulation of PtdSer biosynthesis during apoptosis.

In conclusion, our study has provided novel observations regarding the regulation of PtdSer synthesis and externalization and the involvement of key enzymes in these two events in modulating the progression of apoptosis. These open the possibility for future studies on many aspects of the regulation of PtdSer biosynthesis and activation of PtdSer externalization. Further work on signaling events that may lead to alteration of apoptotic processes could provide novel means for potential therapeutic intervention of apoptosis.

VII. References

Adams, J.M. and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Adams, J.M. and Cory, S. (2001). Life-or-death decisions by the Bcl-2 protein family. Trends Biochem. Sci. *26*, 61-66.

Aderem, A. and Underhill, D.M. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. *17*, 593-623.

Adrain, C. and Martin, S.J. (2001). The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Trends Biochem. Sci. *26*, 390-397.

Amaducci, L., Crook, T.H., Lippi, A., Bracco, L., Baldereschi, M., Latorraca, S., Piersanti, P., Tesco, G., and Sorbi, S. (1991). Use of phosphatidylserine in Alzheimer's disease. Ann. N. Y. Acad. Sci. *640*, 245-249.

Ashkenazi, A. and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science *281*, 1305-1308.

Aussel, C., Breittmayer, J.P., Pelassy, C., and Bernard, A. (1995a). Calmodulin, a junction between two independent immunosuppressive pathways in Jurkat T cells. J. Biol. Chem. *270*, 8032-8036.

Aussel, C., Pelassy, C., and Bernard, A. (1995b). Oleylamine and stearylamine increase phosphatidylserine synthesis in T cells by synergy with calcium ions. Int. J. Biochem. Cell Biol. *27*, 597-602.

Aussel, C., Pelassy, C., and Breittmayer, J.P. (1998). CD95 (Fas/APO-1) induces an increased phosphatidylserine synthesis that precedes its externalization during programmed cell death. FEBS Lett. *431*, 195-199.

Aussel, C., Pelassy, C., Mary, D., Choquet, D., and Rossi, B. (1990). Regulation of interleukin-2 production and phosphatidylserine synthesis in Jurkat T lymphocytes by K^+ channel antagonists. Immunopharmacology *20*, 97-103.

Balasubramanian, K., Bevers, E.M., Willems, G.M., and Schroit, A.J. (2001). Binding of annexin V to membrane products of lipid peroxidation. Biochemistry *40*, 8672-8676.

Balasubramanian, K., Chandra, J., and Schroit, A.J. (1997). Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition. J. Biol. Chem. *272*, 31113-31117.

Balasubramanian, K. and Schroit, A.J. (1998). Characterization of phosphatidylserine-dependent beta2-glycoprotein I macrophage interactions. Implications for apoptotic cell clearance by phagocytes. J. Biol. Chem. *273*, 29272-29277.

Baranska, J. (1989). Mechanism of the ATP-dependent phosphatidylserine synthesis in liver subcellular fractions. FEBS Lett. *256*, 33-37.

Baranska, J. and Grabarek, Z. (1979). Rat liver proteins binding and transferring phosphatidylserine. FEBS Lett. *104*, 253-257.

Basse, F., Stout, J.G., Sims, P.J., and Wiedmer, T. (1996). Isolation of an erythrocyte membrane protein that mediates Ca^{2+}-dependent transbilayer movement of phospholipid. J. Biol. Chem. *271*, 17205-17210.

Bell, R.M. and Coleman, R.A. (1980). Enzymes of glycerolipid synthesis in eukaryotes. Annu. Rev. Biochem. *49*, 459-487.

Bernardi, P., Petronilli, V., Di Lisa, F., and Forte, M. (2001). A mitochondrial perspective on cell death. Trends Biochem. Sci. *26*, 112-117.

Bevers, E.M., Comfurius, P., Dekkers, D.W., and Zwaal, R.F. (1999). Lipid translocation across the plasma membrane of mammalian cells. Biochim. Biophys. Acta *1439*, 317-330.

Bevers, E.M., Comfurius, P., van Rijn, J.L., Hemker, H.C., and Zwaal, R.F. (1982). Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur. J. Biochem. *122*, 429-436.

Bevers, E.M., Comfurius, P., and Zwaal, R.F. (1996). Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications. Lupus *5*, 480-487.

Bezombes, C., Maestre, N., Laurent, G., Levade, T., Bettaieb, A., and Jaffrezou, J.P. (1998). Restoration of TNF-alpha-induced ceramide generation and apoptosis in resistant human leukemia KG1a cells by the P-glycoprotein blocker PSC833. FASEB J. *12*, 101-109.

Bitbol, M. and Devaux, P.F. (1988). Measurement of outward translocation of phospholipids across human erythrocyte membrane. Proc. Natl. Acad. Sci. U. S. A *85*, 6783-6787.

Bjerve, K.S. (1973). The Ca^{2+}-dependent biosynthesis of lecithin, phosphatidyl-ethanolamine and phosphatidylserine in rat liver subcellular particles. Biochim. Biophys. Acta *296*, 549-562.

Bjerve, K.S. (1985). The biosynthesis of phosphatidylserine and phosphatidylethanolamine from L-[3-^{14}C]serine in isolated rat hepatocytes. Biochim. Biophys. Acta *833*, 396-405.

Blankenberg, F.G., Katsikis, P.D., Tait, J.F., Davis, R.E., Naumovski, L., Ohtsuki, K., Kopiwoda, S., Abrams, M.J., Darkes, M., Robbins, R.C., Maecker, H.T., and Strauss,

H.W. (1998). In vivo detection and imaging of phosphatidylserine expression during programmed cell death. Proc. Natl. Acad. Sci. U. S. A. *95*, 6349-6354.

Borkenhagen, J.D., Kennedy, E.P., and Fielding, L. (1961). Enzymatic formation and decarboxylation of phosphatidylserine. J. Biol. Chem. *236*, PC28-PC29.

Borst, P., Zelcer, N., and van Helvoort, A. (2000). ABC transporters in lipid transport. Biochim. Biophys. Acta *1486*, 128-144.

Bortner, C.D. and Cidlowski, J.A. (2002). Cellular mechanisms for the repression of apoptosis. Annu. Rev. Pharmacol. Toxicol. *42*, 259-281.

Bossy-Wetzel, E., Newmeyer, D.D., and Green, D.R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J. *17*, 37-49.

Bratton, D.L. (1994). Polyamine inhibition of transbilayer movement of plasma membrane phospholipids in the erythrocyte ghost. J. Biol. Chem. *269*, 22517-22523.

Bratton, D.L., Fadok, V.A., Richter, D.A., Kailey, J.M., Guthrie, L.A., and Henson, P.M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J. Biol. Chem. *272*, 26159-26165.

Bratton, S.B. and Cohen, G.M. (2001). Apoptotic death sensor: an organelle's alter ego? Trends Pharmacol. Sci. *22*, 306-315.

Bretscher, M.S. (1972). Asymmetrical lipid bilayer structure for biological membranes. Nat. New Biol. *236*, 11-12.

Buratta, S., Fettucciari, K., Mambrini, R., Fetriconi, I., Marconi, P., and Mozzi, R. (2002). Group B streptococcus (GBS) modifies macrophage phosphatidylserine metabolism during induction of apoptosis. FEBS Lett. *520*, 68-72.

Burns, T.F. and El Deiry, W.S. (1999). The p53 pathway and apoptosis. J. Cell Physiol *181*, 231-239.

Carafoli, E. and Molinari, M. (1998). Calpain: a protease in search of a function? Biochem. Biophys. Res. Commun. *247*, 193-203.

Chen, J.S. and Mehta, K. (1999). Tissue transglutaminase: an enzyme with a split personality. Int. J. Biochem. Cell Biol. *31*, 817-836.

Chiu, D., Lubin, B., Roelofsen, B., and van Deenen, L.L. (1981). Sickled erythrocytes accelerate clotting in vitro: an effect of abnormal membrane lipid asymmetry. Blood *58*, 398-401.

Cohen, G.M. (1997). Caspases: the executioners of apoptosis. Biochem. J. *326 (Pt 1)*, 1-16.

Colbeau, A., Nachbaur, J., and Vignais, P.M. (1971). Enzymic characterization and lipid composition of rat liver subcellular membranes. Biochim. Biophys. Acta *249*, 462-492.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. *3*, 339-345.

Comfurius, P., Senden, J.M., Tilly, R.H., Schroit, A.J., Bevers, E.M., and Zwaal, R.F. (1990). Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim. Biophys. Acta *1026*, 153-160.

Connor, J., Pak, C.C., and Schroit, A.J. (1994). Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. J. Biol. Chem. *269*, 2399-2404.

Connor, J., Pak, C.H., Zwaal, R.F., and Schroit, A.J. (1992). Bidirectional transbilayer movement of phospholipid analogs in human red blood cells. Evidence for an ATP-dependent and protein-mediated process. J. Biol. Chem. *267*, 19412-19417.

Crawford, A.M., Kerr, J.F., and Currie, A.R. (1972). The relationship of acute mesodermal cell death to the teratogenic effects of 7-OHM-12-MBA in the foetal rat. Br. J. Cancer *26*, 498-503.

Crook, T.H., Tinklenberg, J., Yesavage, J., Petrie, W., Nunzi, M.G., and Massari, D.C. (1991). Effects of phosphatidylserine in age-associated memory impairment. Neurology *41*, 644-649.

Cucuianu, M., Plesca, L., Bodizs, G., Colhon, D., and Brudasca, I. (1996). Acute phase reaction and the hemostatic balance. Rom. J. Intern. Med. *34*, 13-18.

Cullis, P.R., Fenske, D.B., and Hope, M.J. (1996). Physical properties and functional roles of lipids in membrane. In Biochemistry of Lipids, Lipoproteins and Membranes, D.E.Vance and J.Vance, eds. (New York: Elsevier Science), pp. 1-33.

Czarny, M., Sabala, P., Ucieklak, A., Kaczmarek, L., and Baranska, J. (1992). Inhibition of phosphatidylserine synthesis by glutamate, acetylcholine, thapsigargin and ionophore A23187 in glioma C6 cells. Biochem. Biophys. Res. Commun. *186*, 1582-1587.

Daleke, D.L. and Lyles, J.V. (2000). Identification and purification of aminophospholipid flippases. Biochim. Biophys. Acta *1486*, 108-127.

de Jong, K., Geldwerth, D., and Kuypers, F.A. (1997). Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes. Biochemistry *36*, 6768-6776.

Dekkers, D.W., Comfurius, P., Schroit, A.J., Bevers, E.M., and Zwaal, R.F. (1998a). Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1). Biochemistry *37*, 14833-14837.

Dekkers, D.W., Comfurius, P., van Gool, R.G., Bevers, E.M., and Zwaal, R.F. (2000). Multidrug resistance protein 1 regulates lipid asymmetry in erythrocyte membranes. Biochem. J. *350 Pt 2*, 531-535.

Dekkers, D.W., Comfurius, P., Vuist, W.M., Billheimer, J.T., Dicker, I., Weiss, H.J., Zwaal, R.F., and Bevers, E.M. (1998b). Impaired Ca^{2+}-induced tyrosine phosphorylation and defective lipid scrambling in erythrocytes from a patient with Scott syndrome: a study using an inhibitor for scramblase that mimics the defect in Scott syndrome. Blood *91*, 2133-2138.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A., and Messing, R.O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. Am. J. Physiol Lung Cell Mol. Physiol *279*, L429-L438.

Dennis, E.A. and Kennedy, E.P. (1972). Intracellular sites of lipid synthesis and the biogenesis of mitochondria. J. Lipid Res. *13*, 263-267.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.

Diaz, C. and Schroit, A.J. (1996). Role of translocases in the generation of phosphatidylserine asymmetry. J. Membr. Biol. *151*, 1-9.

Didier, M., Aussel, C., Pelassy, C., and Fehlmann, M. (1988). IL-1 signaling for IL-2 production in T cells involves a rise in phosphatidylserine synthesis. J. Immunol. *141*, 3078-3080.

Ding, J., Wu, Z., Crider, B.P., Ma, Y., Li, X., Slaughter, C., Gong, L., and Xie, X.S. (2000). Identification and functional expression of four isoforms of ATPase II, the putative aminophospholipid translocase. Effect of isoform variation on the ATPase activity and phospholipid specificity. J. Biol. Chem. *275*, 23378-23386.

Dygas, A., Przybylek, K., Meljon, A., and Baranska, J. (2000). Serine base-exchange in rat liver nuclei. FEBS Lett. *482*, 205-208.

Earnshaw, W.C., Martins, L.M., and Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. *68*, 383-424.

Ekert, P.G., Silke, J., and Vaux, D.L. (1999). Caspase inhibitors. Cell Death. Differ. *6*, 1081-1086.

Fabisiak, J.P., Kagan, V.E., Ritov, V.B., Johnson, D.E., and Lazo, J.S. (1997). Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. Am. J. Physiol *272*, C675-C684.

Fabisiak, J.P., Kagan, V.E., Tyurina, Y.Y., Tyurin, V.A., and Lazo, J.S. (1998). Paraquat-induced phosphatidylserine oxidation and apoptosis are independent of activation of PLA2. Am. J. Physiol *274*, L793-L802.

Fadeel, B., Gleiss, B., Hogstrand, K., Chandra, J., Wiedmer, T., Sims, P.J., Henter, J.I., Orrenius, S., and Samali, A. (1999b). Phosphatidylserine exposure during apoptosis is a cell-type-specific event and does not correlate with plasma membrane phospholipid scramblase expression. Biochem. Biophys. Res. Commun. *266*, 504-511.

Fadeel, B., Orrenius, S., and Zhivotovsky, B. (1999a). Apoptosis in human disease: a new skin for the old ceremony? Biochem. Biophys. Res. Commun. *266*, 699-717.

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., and Henson, P.M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature *405*, 85-90.

Fadok, V.A. and Chimini, G. (2001). The phagocytosis of apoptotic cells. Semin. Immunol. *13*, 365-372.

Fadok, V.A. and Henson, P.M. (1998). Apoptosis: getting rid of the bodies. Curr. Biol. *8*, R693-R695.

Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., and Henson, P.M. (1992a). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. *149*, 4029-4035.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992b). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. *148*, 2207-2216.

Farge, E., Ojcius, D.M., Subtil, A., and Dautry-Varsat, A. (1999). Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. Am. J. Physiol *276*, C725-C733.

Folch, J., Lee, M., and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. *226*, 497-509.

Frasch, S.C., Henson, P.M., Kailey, J.M., Richter, D.A., Janes, M.S., Fadok, V.A., and Bratton, D.L. (2000). Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J. Biol. Chem. *275*, 23065-23073.

Galavazi, G. and Bootsma, D. (1966). Synchronization of mammalian cells in vitro by inhibition of the DNA synthesis. II. Population dynamics. Exp. Cell Res. *41*, 438-451.

Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W., and Thornberry, N.A. (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. J. Biol. Chem. *273*, 32608-32613.

Gorczyca, W., Gong, J., Ardelt, B., Traganos, F., and Darzynkiewicz, Z. (1993). The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res. *53*, 3186-3192.

Goyal, L. (2001). Cell death inhibition: keeping caspases in check. Cell *104*, 805-808.

Green, D.R. and Reed, J.C. (1998). Mitochondria and apoptosis. Science *281*, 1309-1312.

Gschwendt, M. (1999). Protein kinase C delta. Eur. J. Biochem. *259*, 555-564.

Hacker, G. (2000). The morphology of apoptosis. Cell Tissue Res. *301*, 5-17.

Haest, C.W., Plasa, G., Kamp, D., and Deuticke, B. (1978). Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. Biochim. Biophys. Acta *509*, 21-32.

Hall, P.A. (1999). Assessing apoptosis: a critical survey. Endocr. Relat Cancer *6*, 3-8.

Hamon, Y., Broccardo, C., Chambenoit, O., Luciani, M.F., Toti, F., Chaslin, S., Freyssinet, J.M., Devaux, P.F., McNeish, J., Marguet, D., and Chimini, G. (2000). ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat. Cell Biol. *2*, 399-406.

Hardardottir, I., Grunfeld, C., and Feingold, K.R. (1995). Effects of endotoxin on lipid metabolism. Biochem. Soc. Trans. *23*, 1013-1018.

Hardardottir, I., Kunitake, S.T., Moser, A.H., Doerrler, W.T., Rapp, J.H., Grunfeld, C., and Feingold, K.R. (1994). Endotoxin and cytokines increase hepatic messenger RNA levels and serum concentrations of apolipoprotein J (clusterin) in Syrian hamsters. J. Clin. Invest *94*, 1304-1309.

Heatwole, V.M. (1999). TUNEL assay for apoptotic cells. Methods Mol. Biol. *115*, 141-148.

Hengartner, M.O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Henson, P.M., Bratton, D.L., and Fadok, V.A. (2001a). Apoptotic cell removal. Curr. Biol. *11*, R795-R805.

Henson, P.M., Bratton, D.L., and Fadok, V.A. (2001b). The phosphatidylserine receptor: a crucial molecular switch? Nat. Rev. Mol. Cell Biol. *2*, 627-633.

Herrmann, A. and Devaux, P.F. (1990). Alteration of the aminophospholipid translocase activity during in vivo and artificial aging of human erythrocytes. Biochim. Biophys. Acta *1027*, 41-46.

Hicks, B.W. and Parsons, S.M. (1992). Characterization of the P-type and V-type ATPases of cholinergic synaptic vesicles and coupling of nucleotide hydrolysis to acetylcholine transport. J. Neurochem. *58*, 1211-1220.

Hoffmann, P.R., deCathelineau, A.M., Ogden, C.A., Leverrier, Y., Bratton, D.L., Daleke, D.L., Ridley, A.J., Fadok, V.A., and Henson, P.M. (2001). Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J. Cell Biol. *155*, 649-660.

Honarpour, N., Du, C., Richardson, J.A., Hammer, R.E., Wang, X., and Herz, J. (2000). Adult Apaf-1-deficient mice exhibit male infertility. Dev. Biol. *218*, 248-258.

Huigsloot, M., Tijdens, I.B., Mulder, G.J., and van de, W.B. (2001). Differential regulation of phosphatidylserine externalization and DNA fragmentation by caspases in anticancer drug-induced apoptosis of rat mammary adenocarcinoma MTLn3 cells. Biochem. Pharmacol. *62*, 1087-1097.

Hübscher, G. (1962). Metabolism of phospholipids VI. The effect of metal ions on the incorporation of L-serine into phosphatidylserine. Biochim. Biophys. Acta *57*, 555-561.

Jackson, M.R., Nilsson, T., and Peterson, P.A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. *9*, 3153-3162.

Jacobson, M.D., Weil, M., and Raff, M.C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

Jelinsky, S.A., Estep, P., Church, G.M., and Samson, L.D. (2000). Regulatory networks revealed by transcriptional profiling of damaged Saccharomyces cerevisiae cells: Rpn4 links base excision repair with proteasomes. Mol. Cell Biol. *20*, 8157-8167.

Jelsema, C.L. and Morre, D.J. (1978). Distribution of phospholipid biosynthetic enzymes among cell components of rat liver. J. Biol. Chem. *253*, 7960-7971.

Johnson, D.E. (2000). Noncaspase proteases in apoptosis. Leukemia *14*, 1695-1703.

Johnson, N., Ng, T.T., and Parkin, J.M. (1997). Camptothecin causes cell cycle perturbations within T-lymphoblastoid cells followed by dose dependent induction of apoptosis. Leuk. Res. *21*, 961-972.

Kagan, V.E., Fabisiak, J.P., Shvedova, A.A., Tyurina, Y.Y., Tyurin, V.A., Schor, N.F., and Kawai, K. (2000). Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. FEBS Lett. *477*, 1-7.

Kamp, D. and Haest, C.W. (1998). Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. Biochim. Biophys. Acta *1372*, 91-101.

Kamp, D., Sieberg, T., and Haest, C.W. (2001). Inhibition and stimulation of phospholipid scrambling activity. Consequences for lipid asymmetry, echinocytosis, and microvesiculation of erythrocytes. Biochemistry *40*, 9438-9446.

Kao, F.T. and Puck, T.T. (1967). Genetics of somatic mammalian cells. IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline. Genetics *55*, 513-524.

Kao, F.T. and Puck, T.T. (1968). Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. U. S. A *60*, 1275-1281.

Kerr, J.F. (1965). A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes. J. Pathol. Bacteriol. *90*, 419-435.

Kerr, J.F. (1969). An electron-microscope study of liver cell necrosis due to heliotrine. J. Pathol. *97*, 557-562.

Kerr, J.F. (1971). Shrinkage necrosis: a distinct mode of cellular death. J. Pathol. *105*, 13-20.

Kerr, J.F. (1972). Shrinkage necrosis of adrenal cortical cells. J. Pathol. *107*, 217-219.

Kerr, J.F. (1999). A personal account of events leading to the definition of the apoptosis concept. Cell Differ. *23*, 1-10.

Kerr, J.F. and Searle, J. (1972). A mode of cell loss in malignant neoplasms. J. Pathol. *106*, xi.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Khwaja, A. and Tatton, L. (1999). Resistance to the cytotoxic effects of tumor necrosis factor alpha can be overcome by inhibition of a FADD/caspase-dependent signaling pathway. J. Biol. Chem. *274*, 36817-36823.

Kidd, V.J., Lahti, J.M., and Teitz, T. (2000). Proteolytic regulation of apoptosis. Semin. Cell Dev. Biol. *11*, 191-201.

Komiyama, T., Quan, L.T., and Salvesen, G.S. (1996). Inhibition of cysteine and serine proteinases by the cowpox virus serpin CRMA. Adv. Exp. Med. Biol. *389*, 173-176.

Kovesdi, I., Brough, D.E., Bruder, J.T., and Wickham, T.J. (1997). Adenoviral vectors for gene transfer. Curr. Opin. Biotechnol. *8*, 583-589.

Krammer, P.H. (2000). CD95's deadly mission in the immune system. Nature *407*, 789-795.

Krueger, A., Baumann, S., Krammer, P.H., and Kirchhoff, S. (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol. Cell Biol. *21*, 8247-8254.

Kuge, O., Hasegawa, K., Saito, K., and Nishijima, M. (1998). Control of phosphatidylserine biosynthesis through phosphatidylserine-mediated inhibition of phosphatidylserine synthase I in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. U. S. A *95*, 4199-4203.

Kuge, O. and Nishijima, M. (1997). Phosphatidylserine synthase I and II of mammalian cells. Biochim. Biophys. Acta *1348*, 151-156.

Kuge, O., Nishijima, M., and Akamatsu, Y. (1985). Isolation of a somatic-cell mutant defective in phosphatidylserine biosynthesis. Proc. Natl. Acad. Sci. U. S. A *82*, 1926-1930.

Kuge, O., Nishijima, M., and Akamatsu, Y. (1986a). Phosphatidylserine biosynthesis in cultured Chinese hamster ovary cells. II. Isolation and characterization of phosphatidylserine auxotrophs. J. Biol. Chem. *261*, 5790-5794.

Kuge, O., Nishijima, M., and Akamatsu, Y. (1986b). Phosphatidylserine biosynthesis in cultured Chinese hamster ovary cells. III. Genetic evidence for utilization of phosphatidylcholine and phosphatidylethanolamine as precursors. J. Biol. Chem. *261*, 5795-5798.

Kuge, O., Nishijima, M., and Akamatsu, Y. (1991). A Chinese hamster cDNA encoding a protein essential for phosphatidylserine synthase I activity. J. Biol. Chem. *266*, 24184-24189.

Kuge, O., Saito, K., and Nishijima, M. (1997). Cloning of a Chinese hamster ovary (CHO) cDNA encoding phosphatidylserine synthase (PSS) II, overexpression of which suppresses the phosphatidylserine biosynthetic defect of a PSS I-lacking mutant of CHO-K1 cells. J. Biol. Chem. *272*, 19133-19139.

Kuge, O., Saito, K., and Nishijima, M. (1999). Control of phosphatidylserine synthase II activity in Chinese hamster ovary cells. J. Biol. Chem. *274*, 23844-23849.

Kuge, O., Yamakawa, Y., and Nishijima, M. (2001). Enhancement of transport-dependent decarboxylation of phosphatidylserine by S100B protein in permeabilized Chinese hamster ovary cells. J. Biol. Chem. *276*, 23700-23706.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., and Flavell, R.A. (1998). Reduced apoptosis and cytochrome C-mediated caspase activation in mice lacking caspase 9. Cell *94*, 325-337.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R.A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature *384*, 368-372.

Kull, F.C., Jr. and Besterman, J.M. (1990). Drug-induced alterations of tumor necrosis factor-mediated cytotoxicity: discrimination of early versus late stage action. J. Cell Biochem. *42*, 1-12.

Kulms, D. and Schwarz, T. (2000). Molecular mechanisms of UV-induced apoptosis. Photodermatol. Photoimmunol. Photomed. *16*, 195-201.

Kushner, I. and Mackiewicz, A. (1987). Acute phase proteins as disease markers. Dis. Markers *5*, 1-11.

Lane, D.P. and Hall, P.A. (1997). MDM2-arbiter of p53's destruction. Trends Biochem. Sci. *22*, 372-374.

Leist, M. and Nicotera, P. (1997). The shape of cell death. Biochem. Biophys. Res. Commun. *236*, 1-9.

Lesort, M., Tucholski, J., Miller, M.L., and Johnson, G.V. (2000). Tissue transglutaminase: a possible role in neurodegenerative diseases. Prog. Neurobiol. *61*, 439-463.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V.M., Adelman, D.M., Simon, M.C., Ma, A., Golden, J.A., Evan, G., Korsmeyer, S.J., MacGregor, G.R., and Thompson, C.B. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell *6*, 1389-1399.

Lorenzo, H.K., Susin, S.A., Penninger, J., and Kroemer, G. (1999). Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death. Differ. *6*, 516-524.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Majno, G. and Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. Am. J. Pathol. *146*, 3-15.

Mandal, D., Moitra, P.K., Saha, S., and Basu, J. (2002). Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. FEBS Lett. *513*, 184-188.

Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993). The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-

dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol. Cell Biol. *13*, 4976-4985.

Mann, C.L., Hughes, F.M., Jr., and Cidlowski, J.A. (2000). Delineation of the signaling pathways involved in glucocorticoid-induced and spontaneous apoptosis of rat thymocytes. Endocrinology *141*, 528-538.

Manno, S., Takakuwa, Y., and Mohandas, N. (2002). Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. Proc. Natl. Acad. Sci. U. S. A *99*, 1943-1948.

Marguet, D., Luciani, M.F., Moynault, A., Williamson, P., and Chimini, G. (1999). Engulfment of apoptotic cells involves the redistribution of membrane phosphatidylserine on phagocyte and prey. Nat. Cell Biol. *1*, 454-456.

Marhaba, R., Dumaurier, M.J., Pelassy, C., Batoz, M., Peyron, J.F., Breittmayer, J.P., and Aussel, C. (1997). The protein tyrosine kinase p56(lck) regulates the serine-base exchange activity in Jurkat T cells. FEBS Lett. *405*, 163-166.

Martelli, A.M., Zweyer, M., Ochs, R.L., Tazzari, P.L., Tabellini, G., Narducci, P., and Bortul, R. (2001). Nuclear apoptotic changes: an overview. J. Cell Biochem. *82*, 634-646.

Martin, S.J., Finucane, D.M., Amarante-Mendes, G.P., O'Brien, G.A., and Green, D.R. (1996). Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J. Biol. Chem. *271*, 28753-28756.

Martin, S.J. and Green, D.R. (1995). Protease activation during apoptosis: death by a thousand cuts? Cell *82*, 349-352.

Martin, S.J., O'Brien, G.A., Nishioka, W.K., McGahon, A.J., Mahboubi, A., Saido, T.C., and Green, D.R. (1995b). Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J. Biol. Chem. *270*, 6425-6428.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995a). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182*, 1545-1556.

Martinez, M.C. and Freyssinet, J.M. (2001). Deciphering the plasma membrane hallmarks of apoptotic cells: Phosphatidylserine transverse redistribution and calcium entry. BMC. Cell Biol. *2*, 20.

Matsumoto, K. (1997). Phosphatidylserine synthase from bacteria. Biochim. Biophys. Acta *1348*, 214-227.

McConkey, D.J. and Orrenius, S. (1996b). Signal transduction pathways in apoptosis. Stem Cells *14*, 619-631.

McConkey, D.J. and Orrenius, S. (1996a). The role of calcium in the regulation of apoptosis. J. Leukoc. Biol. *59*, 775-783.

Meers, P. and Mealy, T. (1993). Calcium-dependent annexin V binding to phospholipids: stoichiometry, specificity, and the role of negative charge. Biochemistry *32*, 11711-11721.

Meier, P., Finch, A., and Evan, G. (2000). Apoptosis in development. Nature *407*, 796-801.

Mesner, P.W., Jr. and Kaufmann, S.H. (1997). Methods utilized in the study of apoptosis. Adv. Pharmacol. *41*, 57-87.

Mikhaevitch, I.S., Singh, I.N., Sorrentino, G., Massarelli, R., and Kanfer, J.N. (1994). Modulation of phosphatidylserine synthesis by a muscarinic receptor occupancy in human neuroblastoma cell line LA-N-1. Biochem. J. *299 (Pt 2)*, 375-380.

Mills, J.C., Stone, N.L., and Pittman, R.N. (1999). Extranuclear apoptosis. The role of the cytoplasm in the execution phase. J. Cell Biol. *146*, 703-708.

Moreau, P. and Cassagne, C. (1994). Phospholipid trafficking and membrane biogenesis. Biochim. Biophys. Acta *1197*, 257-290.

Moriyama, Y. and Nelson, N. (1988). Purification and properties of a vanadate- and N-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. J. Biol. Chem. *263*, 8521-8527.

Morrot, G., Cribier, S., Devaux, P.F., Geldwerth, D., Davoust, J., Bureau, J.F., Fellmann, P., Herve, P., and Frilley, B. (1986). Asymmetric lateral mobility of phospholipids in the human erythrocyte membrane. Proc. Natl. Acad. Sci. U. S. A *83*, 6863-6867.

Morrot, G., Zachowski, A., and Devaux, P.F. (1990). Partial purification and characterization of the human erythrocyte Mg(2+)-ATPase. A candidate aminophospholipid translocase. FEBS Lett. *266*, 29-32.

Mouro, I., Halleck, M.S., Schlegel, R.A., Mattei, M.G., Williamson, P., Zachowski, A., Devaux, P., Cartron, J.P., and Colin, Y. (1999). Cloning, expression, and chromosomal mapping of a human ATPase II gene, member of the third subfamily of P-type ATPases and orthologous to the presumed bovine and murine aminophospholipid translocase. Biochem. Biophys. Res. Commun. *257*, 333-339.

Mozzi, R., Andreoli, V., and Horrocks, L.A. (1993). Phosphatidylserine synthesis in rat cerebral cortex: effects of hypoxia, hypocapnia and development. Mol. Cell Biochem. *126*, 101-107.

Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H., and Mizuno, K. (1996). Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J. Biol. Chem. *271*, 30022-30027.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Naito, M., Nagashima, K., Mashima, T., and Tsuruo, T. (1997). Phosphatidylserine externalization is a downstream event of interleukin-1 beta-converting enzyme family protease activation during apoptosis. Blood *89*, 2060-2066.

Nakano, T., Higashino, K., Kikuchi, N., Kishino, J., Nomura, K., Fujita, H., Ohara, O., and Arita, H. (1995). Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. J. Biol. Chem. *270*, 5702-5705.

Nakano, T., Ishimoto, Y., Kishino, J., Umeda, M., Inoue, K., Nagata, K., Ohashi, K., Mizuno, K., and Arita, H. (1997). Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. J. Biol. Chem. *272*, 29411-29414.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods *139*, 271-279.

Nilsson, K., Forsbeck, K., Gidlund, M., Sundstrom, C., Totterman, T., Sallstrom, J., and Venge, P. (1981). Surface characteristics of the U-937 human histiocytic lymphoma cell line: specific changes during inducible morphologic and functional differentiation in vitro. Hamatol. Bluttransfus. *26*, 215-221.

Nishijima, M., Kuge, O., and Akamatsu, Y. (1986). Phosphatidylserine biosynthesis in cultured Chinese hamster ovary cells. I. Inhibition of de novo phosphatidylserine biosynthesis by exogenous phosphatidylserine and its efficient incorporation. J. Biol. Chem. *261*, 5784-5789.

Nishimoto, I., Okamoto, T., Giambarella, U., and Iwatsubo, T. (1997). Apoptosis in neurodegenerative diseases. Adv. Pharmacol. *41*, 337-368.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science *258*, 607-614.

Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K., and Tabata, S. (1994). Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. DNA Res. *1*, 27-35.

Norbury, C.J. and Hickson, I.D. (2001). Cellular responses to DNA damage. Annu. Rev. Pharmacol. Toxicol. *41*, 367-401.

Olsson, I., Gullberg, U., Ivhed, I., and Nilsson, K. (1983). Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1 alpha, 25-dihydroxycholecalciferol. Cancer Res. *43*, 5862-5867.

Orso, E., Broccardo, C., Kaminski, W.E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M.F., Rothe, G., Lackner, K.J., Chimini, G., and Schmitz, G. (2000). Transport of lipids from Golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. Nat. Genet. *24*, 192-196.

Pastorelli, C., Veiga, J., Charles, N., Voignier, E., Moussu, H., Monteiro, R.C., and Benhamou, M. (2001). IgE receptor type I-dependent tyrosine phosphorylation of phospholipid scramblase. J. Biol. Chem. *276*, 20407-20412.

Pelassy, C., Breittmayer, J.P., and Aussel, C. (1992a). Agonist-induced inhibition of phosphatidylserine synthesis is secondary to the emptying of intracellular Ca^{2+} stores in Jurkat T-cells. Biochem. J. *288 (Pt 3)*, 785-789.

Pelassy, C., Breittmayer, J.P., and Aussel, C. (1999). Regulation of phosphatidylserine synthesis in Jurkat T cell clones: caffeine bypasses CD3/TCR-induced protein tyrosine kinases and calcium signals. Biochem. Biophys. Res. Commun. *266*, 497-503.

Pelassy, C., Breittmayer, J.P., and Aussel, C. (2000b). Inhibition of phosphatidylserine synthesis during Jurkat T cell activation. The phosphatase inhibitor, sodium ortho-vanadate bypasses the CD3/T cell receptor-induced second messenger signaling pathway. Eur. J. Biochem. *267*, 984-992.

Pelassy, C., Breittmayer, J.P., and Aussel, C. (2000a). Regulation of phosphatidylserine exposure at the cell surface by the serine base exchange enzyme system during CD95-induced apoptosis. Biochem. Pharmacol. *59*, 855-863.

Pelassy, C., Breittmayer, J.P., and Aussel, C. (2001). Inhibition of phosphatidylserine synthesis in Jurkat T cells by hydrogen peroxide. Biochim. Biophys. Acta *1539*, 256-264.

Pelassy, C., Breittmayer, J.P., Mary, D., and Aussel, C. (1991). Inhibition of phosphatidylserine synthesis by phosphatidic acid in the Jurkat T cell line: role of calcium ions released from intracellular stores. J. Lipid Mediat. *4*, 199-209.

Pelassy, C., Cattan, N., and Aussel, C. (1992b). Changes in phospholipid metabolism induced by quinine, 4-aminopyridine and tetraethylammonium in the monocytic cell line THP1. Biochem. J. *282 (Pt 2)*, 443-446.

Pelassy, C., Dallanegra, A., Aussel, C., and Fehlmann, M. (1989). Inhibition of phosphatidylserine synthesis induced by triggering CD2 or the CD3-TCR complex in a human T cell line. Relationships with G proteins and receptors modulation. Mol. Immunol. *26*, 1081-1086.

Raggers, R.J., Pomorski, T., Holthuis, J.C., Kalin, N., and van Meer, G. (2000). Lipid traffic: the ABC of transbilayer movement. Traffic. *1*, 226-234.

Rakowska, M. and Wojtczak, L. (1995). Inhibition by glucose and deoxyglucose of phosphatidylserine synthesis in Ehrlich ascites tumor cells: a possible relation to the

Crabtree effect and depletion of endoplasmic reticulum Ca^{2+} stores. Biochem. Biophys. Res. Commun. *207*, 300-305.

Rando, R.R. (1988). Regulation of protein kinase C activity by lipids. FASEB J. *2*, 2348-2355.

Raynal, P. and Pollard, H.B. (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional cal. Biochim. Biophys. Acta *1197*, 63-93.

Reed, J.C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-776.

Reutelingsperger, C.P., Hornstra, G., and Hemker, H.C. (1985). Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. Eur. J. Biochem. *151*, 625-629.

Reutelingsperger, C.P. and van Heerde, W.L. (1997). Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. Cell Mol. Life Sci. *53*, 527-532.

Rich, T., Allen, R.L., and Wyllie, A.H. (2000). Defying death after DNA damage. Nature *407*, 777-783.

Rigotti, A., Acton, S.L., and Krieger, M. (1995). The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. J. Biol. Chem. *270*, 16221-16224.

Rothenberg, M.L. (1997). Topoisomerase I inhibitors: review and update. Ann. Oncol. *8*, 837-855.

Rouser, G., Siakatos, A.N., and Fleisher, S. (1966). Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. Lipids *1*, 85-86.

Rudel, T. and Bokoch, G.M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science *276*, 1571-1574.

Ruetz, S. and Gros, P. (1994). Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell *77*, 1071-1081.

Saito, K., Kuge, O., Akamatsu, Y., and Nishijima, M. (1996). Immunochemical identification of the pssA gene product as phosphatidylserine synthase I of Chinese hamster ovary cells. FEBS Lett. *395*, 262-266.

Saito, K., Nishijima, M., and Kuge, O. (1998). Genetic evidence that phosphatidylserine synthase II catalyzes the conversion of phosphatidylethanolamine to phosphatidylserine in Chinese hamster ovary cells. J. Biol. Chem. *273*, 17199-17205.

Salomoni, P. and Pandolfi, P.P. (2002). p53 de-ubiquitination: at the edge between life and death. Nat. Cell Biol. *4*, E152-E153.

Salvesen, G.S. and Dixit, V.M. (1997). Caspases: intracellular signaling by proteolysis. Cell *91*, 443-446.

Salvesen, G.S. and Dixit, V.M. (1999). Caspase activation: the induced-proximity model. Proc. Natl. Acad. Sci. U. S. A *96*, 10964-10967.

Schlegel, R.A. and Williamson, P. (1987). Membrane phospholipid organization as a determinant of blood cell- reticuloendothelial cell interactions. J. Cell Physiol *132*, 381-384.

Schlegel, R.A. and Williamson, P. (2001). Phosphatidylserine, a death knell. Cell Death. Differ. *8*, 551-563.

Schnaper, H.W. (2000). Signal transduction through protein kinase C. Pediatr. Nephrol. *14*, 254-258.

Schor, N.F., Tyurina, Y.Y., Fabisiak, J.P., Tyurin, V.A., Lazo, J.S., and Kagan, V.E. (1999). Selective oxidation and externalization of membrane phosphatidylserine: Bcl-2-induced potentiation of the final common pathway for apoptosis. Brain Res. *831*, 125-130.

Schroit, A.J. and Zwaal, R.F. (1991). Transbilayer movement of phospholipids in red cell and platelet membranes. Biochim. Biophys. Acta *1071*, 313-329.

Schutze, M.P., Peterson, P.A., and Jackson, M.R. (1994). An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. EMBO J. *13*, 1696-1705.

Scott, R.S., McMahon, E.J., Pop, S.M., Reap, E.A., Caricchio, R., Cohen, P.L., Earp, H.S., and Matsushima, G.K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Seigneuret, M. and Devaux, P.F. (1984). ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc. Natl. Acad. Sci. U. S. A *81*, 3751-3755.

Seigneuret, M., Zachowski, A., Hermann, A., and Devaux, P.F. (1984). Asymmetric lipid fluidity in human erythrocyte membrane: new spin-label evidence. Biochemistry *23*, 4271-4275.

Sheikh, M.S. and Fornace, A.J., Jr. (2000b). Death and decoy receptors and p53-mediated apoptosis. Leukemia *14*, 1509-1513.

Sheikh, M.S. and Fornace, A.J., Jr. (2000a). Role of p53 family members in apoptosis. J. Cell Physiol *182*, 171-181.

Shiao, Y.J., Balcerzak, B., and Vance, J.E. (1998). A mitochondrial membrane protein is required for translocation of phosphatidylserine from mitochondria-associated membranes to mitochondria. Biochem. J. *331 (Pt 1)*, 217-223.

Shiao, Y.J., Lupo, G., and Vance, J.E. (1995). Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. J. Biol. Chem. *270*, 11190-11198.

Shiratsuchi, A., Kawasaki, Y., Ikemoto, M., Arai, H., and Nakanishi, Y. (1999). Role of class B scavenger receptor type I in phagocytosis of apoptotic rat spermatogenic cells by Sertoli cells. J. Biol. Chem. *274*, 5901-5908.

Siddiqui, R.A. and Exton, J.H. (1992). Phospholipid base exchange activity in rat liver plasma membranes. Evidence for regulation by G-protein and P2y-purinergic receptor. J. Biol. Chem. *267*, 5755-5761.

Siegmund, A., Grant, A., Angeletti, C., Malone, L., Nichols, J.W., and Rudolph, H.K. (1998). Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of Saccharomyces cerevisiae. J. Biol. Chem. *273*, 34399-34405.

Silverman, R.H., Halloum, A., Zhou, A., Dong, B., Al Zoghaibi, F., Kushner, D., Zhou, Q., Zhao, J., Wiedmer, T., and Sims, P.J. (2002). Suppression of ovarian carcinoma cell growth in vivo by the interferon-inducible plasma membrane protein, phospholipid scramblase 1. Cancer Res. *62*, 397-402.

Sims, P.J. and Wiedmer, T. (2001). Unraveling the mysteries of phospholipid scrambling. Thromb. Haemost. *86*, 266-275.

Singh, I.N., Massarelli, R., and Kanfer, J.N. (1992a). Modulation of phosphatidylserine homeostasis by amphiphilic cations in a human neuronal cell line, LA-N-2. J. Lipid Mediat. *5*, 301-311.

Singh, I.N., Sorrentino, G., Massarelli, R., and Kanfer, J.N. (1992b). Oleoylamine and sphingosine stimulation of phosphatidylserine synthesis by LA-N-2 cells is protein kinase C independent. FEBS Lett. *296*, 166-168.

Smeets, E.F., Comfurius, P., Bevers, E.M., and Zwaal, R.F. (1994). Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. Biochim. Biophys. Acta *1195*, 281-286.

Smit, J.J., Schinkel, A.H., Oude Elferink, R.P., Groen, A.K., Wagenaar, E., van Deemter, L., Mol, C.A., Ottenhoff, R., van der Lugt, N.M., and van Roon, M.A. (1993). Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell *75*, 451-462.

Smith, A.J., Timmermans-Hereijgers, J.L., Roelofsen, B., Wirtz, K.W., van Blitterswijk, W.J., Smit, J.J., Schinkel, A.H., and Borst, P. (1994). The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett. *354*, 263-266.

Squier, M.K. and Cohen, J.J. (1997). Calpain, an upstream regulator of thymocyte apoptosis. J. Immunol. *158*, 3690-3697.

Squier, M.K., Sehnert, A.J., Sellins, K.S., Malkinson, A.M., Takano, E., and Cohen, J.J. (1999). Calpain and calpastatin regulate neutrophil apoptosis. J. Cell Physiol *178*, 311-319.

Steele, R.J., Thompson, A.M., Hall, P.A., and Lane, D.P. (1998). The p53 tumour suppressor gene. Br. J. Surg. *85*, 1460-1467.

Stone, S.J., Cui, Z., and Vance, J.E. (1998). Cloning and expression of mouse liver phosphatidylserine synthase-1 cDNA. Overexpression in rat hepatoma cells inhibits the CDP- ethanolamine pathway for phosphatidylethanolamine biosynthesis. J. Biol. Chem. *273*, 7293-7302.

Stone, S.J. and Vance, J.E. (1999). Cloning and expression of murine liver phosphatidylserine synthase (PSS)-2: differential regulation of phospholipid metabolism by PSS1 and PSS2. Biochem. J. *342 (Pt 1)*, 57-64.

Stone, S.J. and Vance, J.E. (2000). Phosphatidylserine synthase-1 and -2 are localized to mitochondria- associated membranes. J. Biol. Chem. *275*, 34534-34540.

Stout, J.G., Zhou, Q., Wiedmer, T., and Sims, P.J. (1998). Change in conformation of plasma membrane phospholipid scramblase induced by occupancy of its Ca^{2+} binding site. Biochemistry *37*, 14860-14866.

Strasser, A., O'Connor, L., and Dixit, V.M. (2000). Apoptosis signaling. Annu. Rev. Biochem. *69*, 217-245.

Sturbois-Balcerzak, B., Stone, S.J., Sreenivas, A., and Vance, J.E. (2001). Structure and expression of the murine phosphatidylserine synthase-1 gene. J. Biol. Chem. *276*, 8205-8212.

Sun, J., Nanjundan, M., Pike, L.J., Wiedmer, T., and Sims, P.J. (2002). Plasma Membrane Phospholipid Scramblase 1 Is Enriched in Lipid Rafts and Interacts with the Epidermal Growth Factor Receptor. Biochemistry *41*, 6338-6345.

Sun, J., Zhao, J., Schwartz, M.A., Wang, J.Y., Wiedmer, T., and Sims, P.J. (2001). c-Abl tyrosine kinase binds and phosphorylates phospholipid scramblase 1. J. Biol. Chem. *276*, 28984-28990.

Sundstrom, C. and Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer *17*, 565-577.

Suzuki, T.T. and Kanfer, J.N. (1985). Purification and properties of an ethanolamine-serine base exchange enzyme of rat brain microsomes. J. Biol. Chem. *260*, 1394-1399.

Tait, J.F. and Gibson, D. (1992). Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. Arch. Biochem. Biophys. *298*, 187-191.

Tait, J.F. and Smith, C. (1999). Phosphatidylserine receptors: role of CD36 in binding of anionic phospholipid vesicles to monocytic cells. J. Biol. Chem. *274*, 3048-3054.

Tang, X., Halleck, M.S., Schlegel, R.A., and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity. Science *272*, 1495-1497.

Taniguchi, H. and Manenti, S. (1993). Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. J. Biol. Chem. *268*, 9960-9963.

Tepper, A.D., Ruurs, P., Wiedmer, T., Sims, P.J., Borst, J., and van Blitterswijk, W.J. (2000). Sphingomyelin hydrolysis to ceramide during the execution phase of apoptosis results from phospholipid scrambling and alters cell-surface morphology. J. Cell Biol. *150*, 155-164.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S., and Dixit, V.M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell *81*, 801-809.

Thornberry, N.A. and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-1316.

Thornberry, N.A., Rosen, A., and Nicholson, D.W. (1997). Control of apoptosis by proteases. Adv. Pharmacol. *41*, 155-177.

Tilney, L.G., Harb, O.S., Connelly, P.S., Robinson, C.G., and Roy, C.R. (2001). How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J. Cell Sci. *114*, 4637-4650.

Trump, B.F., Berezesky, I.K., Chang, S.H., and Phelps, P.C. (1997). The pathways of cell death: oncosis, apoptosis, and necrosis. Toxicol. Pathol. *25*, 82-88.

Tschopp, J., Irmler, M., and Thome, M. (1998). Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. *10*, 552-558.

Tyurina, Y.Y., Shvedova, A.A., Kawai, K., Tyurin, V.A., Kommineni, C., Quinn, P.J., Schor, N.F., Fabisiak, J.P., and Kagan, V.E. (2000). Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. Toxicology *148*, 93-101.

Tzang, B.S., Lai, Y.C., Hsu, M., Chang, H.W., Chang, C.C., Huang, P.C., and Liu, Y.C. (1999a). Function and sequence analyses of tumor suppressor gene p53 of CHO.K1 cells. DNA Cell Biol. *18*, 315-321.

Tzang, B.S., Lai, Y.C., and Liu, Y.C. (1999b). UV-induced but p53 independent apoptotic death in CHO.K1 cells is promoted by M phase inhibitors. In Vitro Cell Dev. Biol. Anim *35*, 17-18.

van den Eijnde, S.M., Boshart, L., Baehrecke, E.H., De Zeeuw, C.I., Reutelingsperger, C.P.M., and Vermeij-Keers, C. (1998). Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved. Apoptosis. *3*, 9-16.

van den Eijnde, S.M., van den Hoff, M.J., Reutelingsperger, C.P., van Heerde, W.L., Henfling, M.E., Vermeij-Keers, C., Schutte, B., Borgers, M., and Ramaekers, F.C. (2001). Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation. J. Cell Sci. *114*, 3631-3642.

van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B., and Reutelingsperger, C.P. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry *31*, 1-9.

van Engeland, M., Ramaekers, F.C., Schutte, B., and Reutelingsperger, C.P. (1996). A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry *24*, 131-139.

van Golde, L.M., Raben, J., Batenburg, J.J., Fleischer, B., Zambrano, F., and Fleischer, S. (1974). Biosynthesis of lipids in Golgi complex and other subcellular fractions from rat liver. Biochim. Biophys. Acta *360*, 179-192.

van Meer, G. (1989). Lipid traffic in animal cells. Annu. Rev. Cell Biol. *5*, 247-275.

Vanags, D.M., Porn-Ares, M.I., Coppola, S., Burgess, D.H., and Orrenius, S. (1996). Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. J. Biol. Chem. *271*, 31075-31085.

Vance, D.E., Houweling,M., Lee,M., and Cui,Z. (1996). Phosphatidylethanolamine methylation and hepatoma cell growth. Anticancer Res. *16*, 1413-1416.

Vance, J.E. (1988). Compartmentalization of phospholipids for lipoprotein assembly on the basis of molecular species and biosynthetic origin. Biochim. Biophys. Acta *963*, 70-81.

Vance, J.E. (1989). The use of newly synthesized phospholipids for assembly into secreted hepatic lipoproteins. Biochim. Biophys. Acta *1006*, 59-69.

Vance, J.E. (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. J. Biol. Chem. *265*, 7248-7256.

Vance, J.E. and Vance, D.E. (1986). Specific pools of phospholipids are used for lipoprotein secretion by cultured rat hepatocytes. J. Biol. Chem. *261*, 4486-4491.

Vander Heiden, M.G., Chandel, N.S., Schumacker, P.T., and Thompson, C.B. (1999). Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell *3*, 159-167.

Vandivier, R.W., Fadok, V.A., Hoffmann, P.R., Bratton, D.L., Penvari, C., Brown, K.K., Brain, J.D., Accurso, F.J., and Henson, P.M. (2002). Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. J. Clin. Invest *109*, 661-670.

Verhoven, B., Schlegel, R.A., and Williamson, P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J. Exp. Med. *182*, 1597-1601.

Villa, P., Kaufmann, S.H., and Earnshaw, W.C. (1997). Caspases and caspase inhibitors. Trends Biochem. Sci. *22*, 388-393.

Villa, P.G., Henzel, W.J., Sensenbrenner, M., Henderson, C.E., and Pettmann, B. (1998). Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. J. Cell Sci. *111 (Pt 6)*, 713-722.

Vincent, P., Maneta-Peyret, L., Sturbois-Balcerzak, B., Duvert, M., Cassagne, C., and Moreau, P. (1999). One of the origins of plasma membrane phosphatidylserine in plant cells is a local synthesis by a serine exchange activity. FEBS Lett. *464*, 80-84.

Vincent, P., Sargueil, F., Sturbois-Balcerzak, B., Cassagne, C., and Moreau, P. (2001). Phosphatidylserine increase in rat liver endomembranes during the acute phase response. Biochimie *83*, 957-960.

Voelker, D.R. (1984). Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells. Proc. Natl. Acad. Sci. U. S. A *81*, 2669-2673.

Voelker, D.R. (1991). Adriamycin disrupts phosphatidylserine import into the mitochondria of permeabilized CHO-K1 cells. J. Biol. Chem. *266*, 12185-12188.

Voelker, D.R. (1996). Lipid assembly into cell membranes. In Biochemistry of Lipids, Lipoproteins and Membranes, D.E.Vance and J.Vance, eds. (New York: Elsevier Science), pp. 391-423.

Voelker, D.R. (1997). Phosphatidylserine decarboxylase. Biochim. Biophys. Acta *1348*, 236-244.

Voelker, D.R. (2000). Interorganelle transport of aminoglycerophospholipids. Biochim. Biophys. Acta *1486*, 97-107.

Voelker, D.R. and Frazier, J.L. (1986). Isolation and characterization of a Chinese hamster ovary cell line requiring ethanolamine or phosphatidylserine for growth and exhibiting defective phosphatidylserine synthase activity. J. Biol. Chem. *261*, 1002-1008.

Walker, P.R. and Sikorska, M. (1997). New aspects of the mechanism of DNA fragmentation in apoptosis. Biochem. Cell Biol. *75*, 287-299.

Walkey, C.J., Cui, Z., Agellon, L.B., and Vance, D.E. (1996). Characterization of the murine phosphatidylethanolamine N-methyltransferase-2 gene. J. Lipid Res. *37*, 2341-2350.

Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A.V., and Boldin, M.P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. *17*, 331-367.

Wang, S.Y., Moriyama, Y., Mandel, M., Hulmes, J.D., Pan, Y.C., Danho, W., Nelson, H., and Nelson, N. (1988). Cloning of cDNA encoding a 32-kDa protein. An accessory polypeptide of the H^+-ATPase from chromaffin granules. J. Biol. Chem. *263*, 17638-17642.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. Genes Dev. *15*, 2922-2933.

Waterhouse, N.J., Finucane, D.M., Green, D.R., Elce, J.S., Kumar, S., Alnemri, E.S., Litwack, G., Khanna, K., Lavin, M.F., and Watters, D.J. (1998). Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death. Differ. *5*, 1051-1061.

Webb, S.J., Harrison, D.J., and Wyllie, A.H. (1997). Apoptosis: an overview of the process and its relevance in disease. Adv. Pharmacol. *41*, 1-34.

Weber, C.H. and Vincenz, C. (2001). The death domain superfamily: a tale of two interfaces? Trends Biochem. Sci. *26*, 475-481.

Weil, M., Jacobson, M.D., and Raff, M.C. (1998). Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. J. Cell Sci. *111 (Pt 18)*, 2707-2715.

Wiedmer, T., Zhou, Q., Kwoh, D.Y., and Sims, P.J. (2000). Identification of three new members of the phospholipid scramblase gene family. Biochim. Biophys. Acta *1467*, 244-253.

Wiktorek-Wojcik, M., Banasiak, M., Czarny, M., Stepkowski, D., and Baranska, J. (1997). Serine base exchange enzyme activity is modulated by sphingosine and other amphiphilic compounds: possible role of positive charge in increasing the synthesis of phosphatidylserine. Biochem. Biophys. Res. Commun. *241*, 73-78.

Wiktorek, M., Sabala, P., Czarny, M., and Baranska, J. (1996). Phosphatidylserine synthesis in glioma C6 cells is inhibited by Ca^{2+} depletion from the endoplasmic

reticulum: effects of 2,5-di-tert-butylhydroquinone and thimerosal. Biochem. Biophys. Res. Commun. *224*, 645-650.

Williamson, P., Antia, R., and Schlegel, R.A. (1987). Maintenance of membrane phospholipid asymmetry. Lipid-cytoskeletal interactions or lipid pump? FEBS Lett. *219*, 316-320.

Williamson, P., Bevers, E.M., Smeets, E.F., Comfurius, P., Schlegel, R.A., and Zwaal, R.F. (1995). Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. Biochemistry *34*, 10448-10455.

Williamson, P., Christie, A., Kohlin, T., Schlegel, R.A., Comfurius, P., Harmsma, M., Zwaal, R.F., and Bevers, E.M. (2001). Phospholipid scramblase activation pathways in lymphocytes. Biochemistry *40*, 8065-8072.

Williamson, P., Kulick, A., Zachowski, A., Schlegel, R.A., and Devaux, P.F. (1992). Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. Biochemistry *31*, 6355-6360.

Willingham, M.C. (1999). Cytochemical methods for the detection of apoptosis. J. Histochem. Cytochem. *47*, 1101-1110.

Wolf, B.B., Goldstein, J.C., Stennicke, H.R., Beere, H., Amarante-Mendes, G.P., Salvesen, G.S., and Green, D.R. (1999). Calpain functions in a caspase-independent manner to promote apoptosis- like events during platelet activation. Blood *94*, 1683-1692.

Wolf, B.B. and Green, D.R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. J. Biol. Chem. *274* , 20049-20052.

Wood, D.E. and Newcomb, E.W. (1999). Caspase-dependent activation of calpain during drug-induced apoptosis. J. Biol. Chem. *274* , 8309-8315.

Wood, D.E., Thomas, A., Devi, L.A., Berman, Y., Beavis, R.C., Reed, J.C., and Newcomb, E.W. (1998). Bax cleavage is mediated by calpain during drug-induced apoptosis. Oncogene *17*, 1069-1078.

Wright, S.C., Zheng, H., and Zhong, J. (1996). Tumor cell resistance to apoptosis due to a defect in the activation of sphingomyelinase and the 24 kDa apoptotic protease (AP24). FASEB J. *10*, 325-332.

Wu, Y.C. and Horvitz, H.R. (1998). The C. elegans cell corpse engulfment gene ced-7 encodes a protein similar to ABC transporters. Cell *93*, 951-960.

Xu, Z., Byers, D.M., Palmer, F.B., and Cook, H.W. (1994). Serine and ethanolamine incorporation into different plasmalogen pools: subcellular analyses of phosphoglyceride synthesis in cultured glioma cells. Neurochem. Res. *19*, 769-775.

Yamashita, S. and Nikawa, J. (1997). Phosphatidylserine synthase from yeast. Biochim. Biophys. Acta *1348*, 228-235.

Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M., and Mak, T.W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell *94*, 739-750.

Yu, A., Byers, D.M., Ridgway, N.D., McMaster, C.R., and Cook, H.W. (2000). Preferential externalization of newly synthesized phosphatidylserine in apoptotic U937 cells is dependent on caspase-mediated pathways. Biochim. Biophys. Acta *1487*, 296-308.

Yuan, J. and Yankner, B.A. (2000). Apoptosis in the nervous system. Nature *407*, 802-809.

Zhang, G., Gurtu, V., Kain, S.R., and Yan, G. (1997). Early detection of apoptosis using a fluorescent conjugate of annexin V. Biotechniques *23*, 525-531.

Zhao, J., Zhou, Q., Wiedmer, T., and Sims, P.J. (1998a). Level of expression of phospholipid scramblase regulates induced movement of phosphatidylserine to the cell surface. J. Biol. Chem. *273*, 6603-6606.

Zhao, J., Zhou, Q., Wiedmer, T., and Sims, P.J. (1998b). Palmitoylation of phospholipid scramblase is required for normal function in promoting Ca^{2+}-activated transbilayer movement of membrane phospholipids. Biochemistry *37*, 6361-6366.

Zhou, Q., Sims, P.J., and Wiedmer, T. (1998). Identity of a conserved motif in phospholipid scramblase that is required for Ca^{2+}-accelerated transbilayer movement of membrane phospholipids. Biochemistry *37*, 2356-2360.

Zhou, Q., Zhao, J., Al Zoghaibi, F., Zhou, A., Wiedmer, T., Silverman, R.H., and Sims, P.J. (2000). Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon-alpha. Blood *95*, 2593-2599.

Zhou, Q., Zhao, J., Stout, J.G., Luhm, R.A., Wiedmer, T., and Sims, P.J. (1997). Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. J. Biol. Chem. *272*, 18240-18244.

Zhou, Q., Zhao, J., Wiedmer, T., and Sims, P.J. (2002). Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. Blood *99*, 4030-4038.

Zhuang, J., Ren, Y., Snowden, R.T., Zhu, H., Gogvadze, V., Savill, J.S., and Cohen, G.M. (1998). Dissociation of phagocyte recognition of cells undergoing apoptosis from other features of the apoptotic program. J. Biol. Chem. *273*, 15628-15632.

Zwaal, R.F. and Schroit, A.J. (1997). Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood *89*, 1121-1132.