

Regulation of Cardiolipin Biosynthesis By Phospholipid Scramblase-3

By

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A Thesis submitted to the Faculty of Graduate
Studies of

The University of Manitoba

in partial fulfilment of the requirements of the
degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics

University of Manitoba

Winnipeg

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Cardiolipin is an important phospholipid synthesized in the inner mitochondrial membrane. It is transported to the outer mitochondrial membrane by a newly described protein phospholipid scramblase-3 (PLS3). The purpose of this project was to assess if biosynthesis of cardiolipin was influenced by varying the levels of functional PLS3. HeLa cells expressing disrupted PLS3 (F258V) or overexpressing PLS3 (PLSCR3) or control were incubated with [³H]glycerol and incorporation of radioactivity into cardiolipin was determined. Incorporation of [³H]glycerol into cardiolipin increased 2.1-fold in F258V cells and 1.8-fold in PLSCR3 cells compared to control. This coincided with a 2.6-fold increase in cardiolipin synthase activity in F258V cells and a 1.6-fold increase in cardiolipin synthase activity in PLSCR3 cells compared to control. There was also a 0.78-fold and 0.52-fold increase in relative level of CL synthase RNA in F258V and PLSCR3 cells, respectively, compared to control. In contrast, [¹⁴C]linoleic acid incorporation into cardiolipin, a measure of cardiolipin remodeling, was reduced 0.45-fold in F258V cells and 0.52-fold in PLSCR3 compared to control. This was due to a decrease in mitochondrial monolysocardiolipin acyltransferase activity in both F258V and PLSCR3 cells compared to control. [³H]Serine incorporation into mitochondrial phosphatidylethanolamine was unaltered, indicating the effect of altered functional levels of PLS3 was exclusive to cardiolipin. In summary, the data indicate that changes in cardiolipin synthesis may be linked to altered

levels of PLS-3. Hence, PLS3 may be an important regulator of cardiolipin *de novo* biosynthesis, and an increase in cardiolipin biosynthesis may be a compensatory response to the modified trafficking of mitochondrial cardiolipin.

ACKNOWLEDGEMENTS

This work was supported by operating grants from the Heart and Stroke Foundation of Manitoba and the Canadian Institutes of Health Research. I would like to thank my supervisor Dr. Grant M. Hatch for his guidance throughout the project. I would also like to thank William A Taylor and Fred Y Xu who taught and helped me with the protocols of all the performed experiments.

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List of Abbreviations

Cardiolipin: CL
Cardiolipin synthase: CLS
CDP-DG synthase: CDS
Cytidine-5'-diphosphate -1,2-diacylglycerol: CDP-DG
Cytidine triphosphate: CTP
Endoplasmic reticulum: ER
Glycerol-3-phosphate: G3P
Lysophosphatidylethanolamine acyltransferase: LPE AT
Lysophosphatidylglycerol acyltransferase: LPG AT
Mitochondrial membrane permeabilization: MMP
Monolysocardiolipin: MLCL
Monolysocardiolipin acyltransferase: MLCL AT
N-terminal Bid: n-Bid
PGP phosphatase: PGPP
PGP synthase: PGPS
Phosphatidic acid: PA
Phosphatidylcholine: PC
Phosphatidylethanolamine: PE
Phosphatidylglycerol: PG
Phosphatidylglycerolphosphate: PGP
Phosphatidylinositol: PI
Phosphatidylserine: PS
Phospholipase A₂: PLA₂
Phospholipid scramblase: PLS
Phospholipid scramblase-3: PLS3
PLS3-disrupted: F258V
PLS3-overexpressed: PLSCR3
Protein kinase C- δ : PKC δ
Truncated Bid: t-Bid

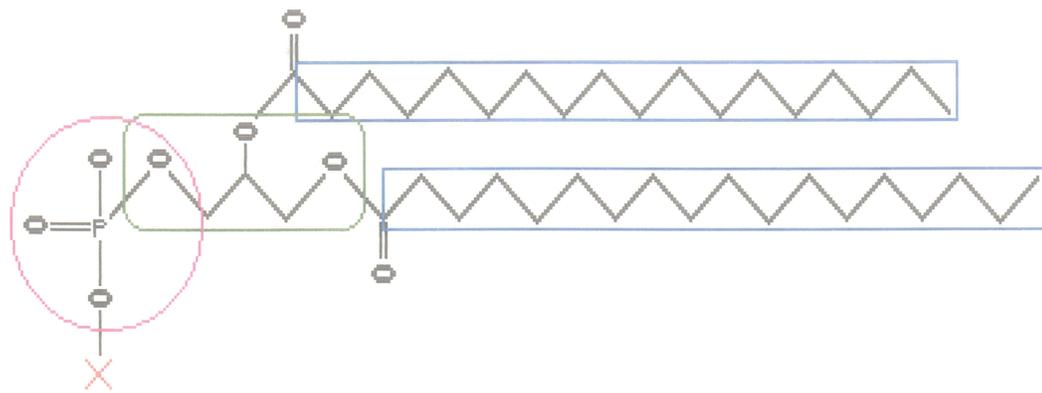
INTRODUCTION

Phospholipid structure and function

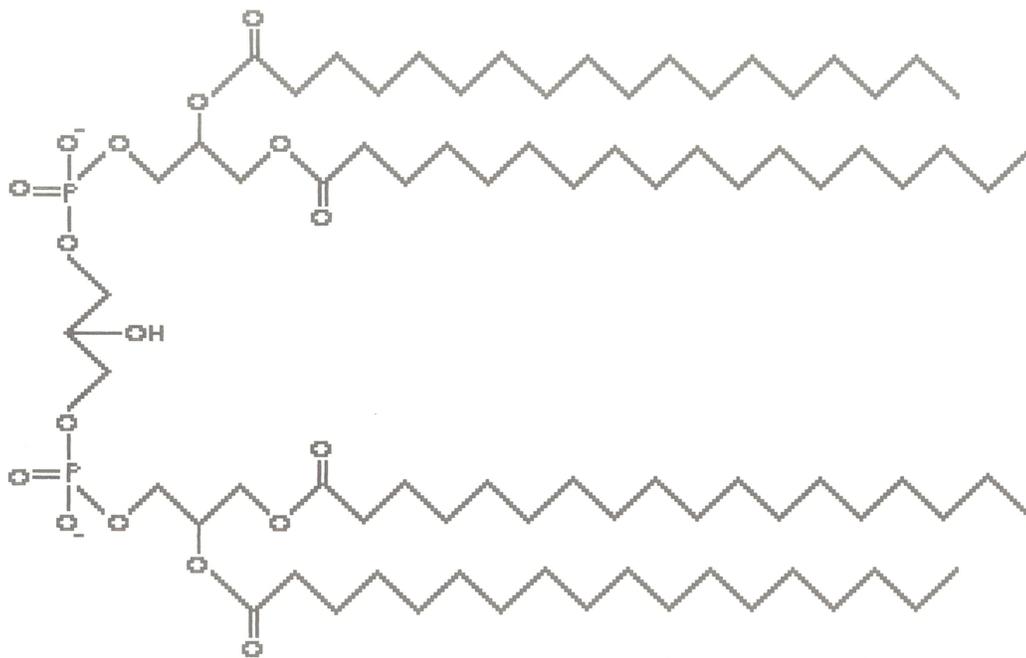
Phospholipids are the principal functional and structural components of the biological membrane (Alberts et al., 1983). Phospholipids are small amphipathic molecules where glycerol is bonded to two fatty acids, acting as the hydrophobic end, and a phosphate group. This phosphate group in turn is attached to a distinct hydrophilic head group that differentiates the various phospholipids. The fatty acids vary in length from 14 to 24 carbon atoms. Approximately half of the fatty acids contain one or more *cis*-double bonds (unsaturated). The degree of unsaturation of the tails influences the fluidity of the membrane. Cell membranes contain a variety of different phospholipids that are distributed asymmetrically throughout the bilayer. Phospholipids form organelles and behave as a protective membrane barrier for the cell.

Cardiolipin structure

Polyglycerophospholipids are a class of phospholipids that have 2 or 3 glycerol molecules linked by phosphodiester bonds, and contain 2 to 4 fatty acid chains of various species and composition (Hatch et al., 2004). The major polyglycerophospholipid in mammalian tissues is bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol or cardiolipin (CL) (Hostetler 1982) (Fig. 1). CL comprises 15% of total phospholipids in rat heart (Hatch, 1994), and comprises 21% of the phospholipid mass of the inner mitochondrial membrane (Krebs et



A



B

Figure 1: Typical structure of a phospholipid. A: Phospholipid containing two fatty acid chains, a glycerol and a phosphate group, X represents various head groups differentiating numerous phospholipids. B: Structure of cardiolipin, with 4 fatty acid chains, 3 glycerol molecules, and 2 phosphate groups.

al., 1979). Studies have determined that 43% of CL faces the matrix side of the inner membrane of the mitochondria while 57% is located on the cytoplasmic side (Cheneval et al., 1988). Eukaryotic cardiolipin acyl positions are occupied by monounsaturated and diunsaturated C16-C18 chains (Schlame et al., 1993). The dominant chain in mammalian membranes are linoleoyl (18:2) and to a lesser extent oleoyl groups (16:1) (Hostetler 1982). CL models demonstrate that its phosphates form a tight bicyclic structure with a hydrogen bond to the hydroxyl on the linking glycerol by trapping a proton, therefore forming an acid-anion (Kates et al., 1993).

Cardiolipin biosynthesis and regulation

The *de novo* biosynthesis of CL in the inner mitochondria occurs via the cytidine-5'-diphosphate-1,2-diacylglycerol (CDP-DG) pathway (Fig. 2) (Hatch 1994). Initially, phosphatidic acid (PA) is converted to CDP-DG by CDP-DG synthase (CDS), which is a rate-limiting step of the pathway (Hatch, 1994). CDP-DG then condenses with glycerol-3-phosphate to form phosphatidylglycerolphosphate (PGP) catalyzed by phosphatidylglycerolphosphate (PGP) synthase, the committed step of the pathway. PGP is then quickly converted to phosphatidylglycerol (PG) by PGP phosphatase. In the last step of the pathway, PG is converted to CL by

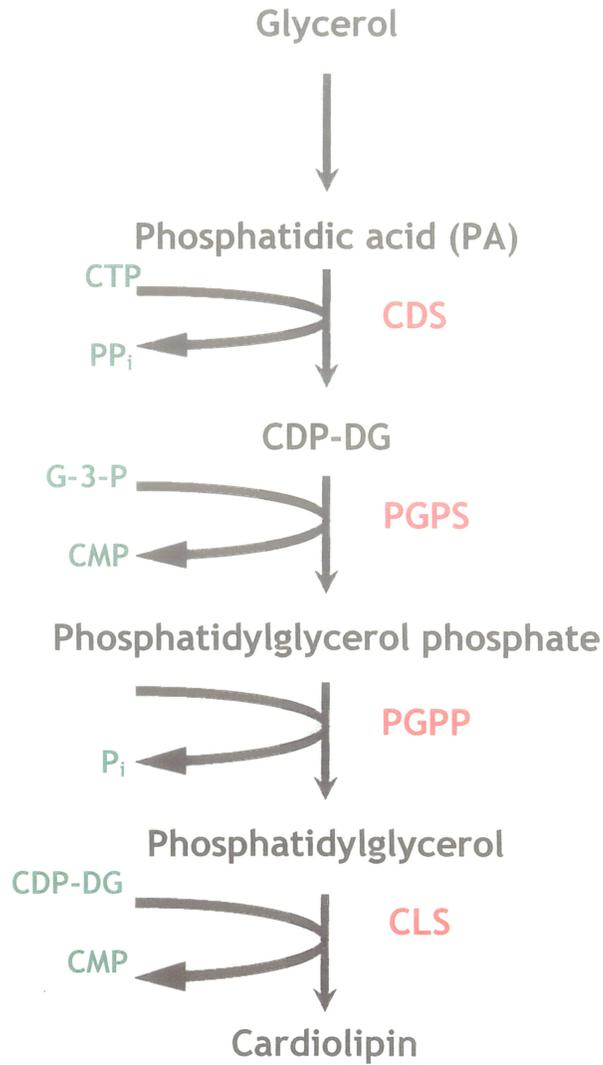


Figure 2: The biosynthesis of cardiolipin, following a cytidine-diphosphate-diacylglycerol (CDP-DG) pathway. Abbreviations: CDS, CDP-DG synthase; PGPS, phosphatidylglycerol phosphate synthase; G-3-P, glycerol-3-phosphate; PGPP, phosphatidylglycerol phosphate phosphatase; CLS; cardiolipin synthase.

condensation with CDP-DG catalyzed by CL synthase. CL and PG synthesis occur in the mitochondria (Hatch, 1994; Hostetler and Van den Bosch 1972; Schlame and Haldar 1993), although PG may also be synthesized in microsomes (Hostetler and Van den Bosch 1972). PA is formed in the outer mitochondrial membrane and the endoplasmic reticulum, in addition to the inner mitochondrial membrane (Hostetler et al, 1982). PGP synthase and PGP phosphatase are found to be essential components of the inner mitochondrial membrane, which regulate levels of PGP and ultimately CL for growth and survival (Hatch 2004). CDP-DG synthase and CL synthase are also associated with the inner mitochondrial membrane where the hydrophilic domains are exposed to the matrix side, therefore newly synthesized CL, PG and CDP-DG in the inner membrane are accessible to newly imported protein in the matrix (Schlame and Haldar 1993). CL is then quickly remodeled by deacylation catalyzed by phospholipase A₂ (PLA₂) to form monolysocardiolipin (MLCL) and then reacylation using linoleoyl-CoA or oleoyl-CoA as substrate, catalyzed by either an endoplasmic reticulum (ER) or mitochondrial MLCL acyltransferase (MLCL AT) or a CL transacylase to form the new CL (Figure 3) (Hauff and Hatch, 2006, Schlame and Rustow, 1990, Ma et al., 1999). Remodeling of CL is important to attain the correct acyl composition needed for its ability to bind to and stimulate proteins in the mitochondrial membrane, given that the fatty acid arrangement of its precursors is different from remodeled CL (Vik, 1981). Mitochondrial MLCL AT displays a molecular mass of 74 kDa and a ping pong

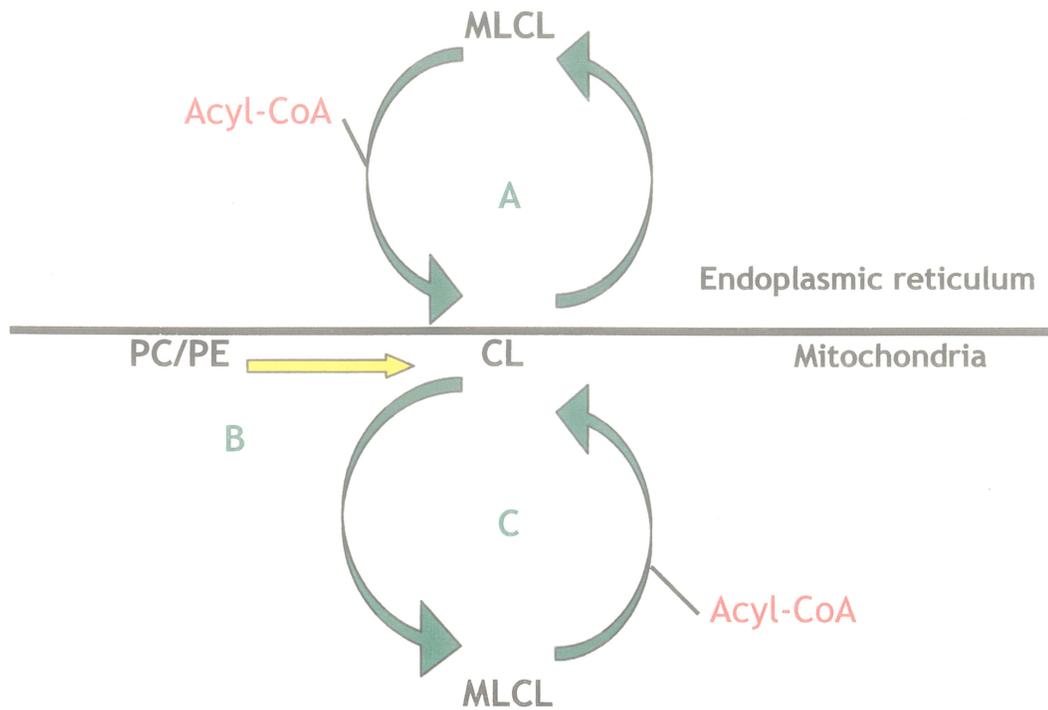


Figure 3: Pathways of cardiolipin remodeling. (A) ER MLCL AT for CL resynthesis in the ER with acyl-CoA as a cofactor in the transfer of an acyl chain to MLCL. (B) mitochondrial CL transacylase requires PC or PE as an acyl donor. (C) mitochondrial MLCL AT requires acyl-CoA as a cofactor in the transfer of an acyl chain to MLCL. In (A) and (C) CL is first deacylated to MLCL by phospholipase A₂.

reaction mechanism with substrate specificity to MLCL (Taylor and Hatch, 2003).

The CL synthesis pathway is regulated by factors that affect phospholipid biosynthesis, mitochondrial development, and cytochrome oxidase assembly (Jiang et al., 1999). To understand this regulation, it is wise to investigate the precursors and their enzymes. PGS1 (chromosome III) and CRD1 (chromosome IV) were discovered to code for the enzymes PGP synthase and CL synthase, respectively, from the *Saccharomyces* Genome Database (Chang et al., 1998a,b). PGS1 transcription is regulated by expression of a 284bp reporter gene fused to a 5' regulatory region, where expression is reduced when exposed to inositol (Chang et al., 1998b). Another way inositol influence PGP synthase activity is by triggering phosphorylation of the enzyme to decrease its activity (He and Greenberg, 2004). CRD1 gene expression, however, is not affected by inositol, but instead controlled by factors affecting mitochondrial development and is crucial for growth at elevated temperatures (Jiang et al., 1999). Stationary growth phase, presence of mitochondrial genome, and non-fermentable carbon sources are some of these conditions that instigate derepression of CRD1 expression (Schlame et al. 2000). CL synthase requires Co^{2+} as a cofactor, presence of phosphatidylethanolamine and alkaline pH (Schlame and Haldar, 1993) for activation, and is inhibited by cations such as Cd^{2+} , Zn^{2+} , Ca^{2+} and Ba^{2+} (McMurray and Jarvis, 1980). CL synthase activity is also dependent on the assembly of the mitochondrial membrane respiratory complex IV, where studies demonstrated mutants of cytochrome c oxidase

showed significantly decreased CL synthase activity (Zhao et al., 1998). CL synthase activity is also dependent on the pH of the respiratory electron transport chain (Gohil et al., 2004). Synthesis of CDP-DG, the rate-limiting step, can be regulated by the inhibitory enzyme CDP-DG hydrolase (Nicholson and McMurray 1984). CL biosynthesis normally draws PG precursors from the mitochondria, but can also utilize exogenous PG sources, therefore mechanisms that monitor the trafficking of different pools of PG into the mitochondria may regulate the production of CL (Hatch et al, 1995). Levels of ATP and CTP also regulate CL synthesis, given that production of CDP-DG requires CTP, and ATP is needed to produce CTP (Hatch and McClarty, 1996). Thyroid hormones also affect the phospholipid composition in the mitochondrial membrane. An increase in CL and PG synthesis and activity of PGP synthase and CL synthase are observed with elevated thyroid states (Cao et al., 1995). Studies have demonstrated that MLCL AT activity may be regulated by CL synthase activity and CL content in the membrane, where an increase or decrease in CL and CL synthase activity were accompanied by an increase or decrease in MLCL AT activity (Taylor et al., 2002). The importance of acyltransferases such as MLCL AT for CL is evident in Barth syndrome, an X-linked disease caused by a mutation of tafazzin (Valianpour et al., 2005). Given that tafazzin is an acyltransferase, mutations causing abnormal acyl conformations in CL may result in symptoms such as dilated cardiomyopathy, skeletal myopathy, neutropenia, aciduria, abnormal mitochondria, and growth retardation (Barth et al., 1999, Gonzalez, 2005).

Cardiolipin function

Cardiolipin engages in the formation of structures in the biological membrane, which play a role in membrane assembly, transmembrane protein movement, or contact-site formation (Schlame et al., 2000). CL deficiency leads to alteration in the stability and fluidity of the mitochondrial membrane (Pfeiffer et al., 2003). Other outcomes include reduced respiratory control and coupling efficiency of oxidative phosphorylation, decreased resistance to elevated temperature and a compromised ability to restore membrane potential (Koshkin and Greenberg, 2000, Ostrander et al., 2001). CL also interacts with numerous proteins present in the inner membrane, involving a nonchemical bonding and functional activation. CL displays nonselective protein binding, yet many of these proteins prefer CL to other phospholipids. CL is believed to play a role in *E. coli* DNA replication initiation, where it binds and activate DNAa, a protein required to initiate replication (Xia and Dowhan, 1995). CL has also been shown to diminish the activity of mitochondrial Ca^{2+} uniporter (Zazueta et al., 2003). The function of calcium transporters are influenced by the fluidity of the mitochondrial membrane, which is affected by the concentration of CL in the lipid bilayer. CL is responsible for modulation of the activity of a number of mitochondrial membrane enzymes involved in the generation of ATP (for reviews see Hoch 1992; Hatch 1998, 2004). The ATP-ADP carrier protein activity depends on the tight bond with 6 molecules of CL, where the diphosphatidylglycerol head group and the four acyl chains are imperative for the firm protein-phospholipid interaction (Beyer and Nuscher,

1996). Once bound with CL, the carrier then undergoes the conformational change from the cytosolic-state to the matrix-state with the addition of ATP. Cleavage of the protein-associated CL by phospholipase A₂ causes the protein to enter a labile state and to display a complete loss of activity, thereby indicating the necessity of CL for the stabilization of the ATP-ADP carrier protein (Beyer and Nuscher, 1996). Creatine kinase has a binding site for CL in the outer leaflet of the inner mitochondrial membrane (Cheneval and Carafoli, 1988). Since creatine kinase ultimately accepts ATP from the ATP-ADP carrier, a close association between the carrier and the enzyme via CL is vital. CL has an inhibitory effect on the activity of carnitine palmitoyltransferase when bound to this enzyme (Fiol and Bieber, 1984; Pande et al., 1986). CL is essential in the function and stabilization of respiratory complexes I (NADH-ubiquinone reductase) (Zhao et al., 1998), III (ubiquinol:cytochrome c oxidoreductase) and IV (cytochrome c oxidase) (Pfeiffer et al., 2003). The electrochemical proton gradient created by these complexes across the inner membrane is used to produce ATP by CL-bound complex V (F₁F₀-ATP synthase). Reactive oxygen species, which are free radicals produced by the mitochondrial electron transport chain, have been demonstrated to affect complex I activity by causing oxidative damage to CL (Paradies et al., 2002). Complex III has CL securely bound to the opening created by cytochrome b and c₁ helices, with the CL head group near the quinone reduction site (Q_i). In rat heart subjected to ischemia and reperfusion the reduction in complex III activity is coupled with a reduction in CL (Petrosillo et al., 2003). Studies have shown that complex IV

requires 2-4 molecules of CL needed to stimulate one cytochrome c oxidase molecule for maximal electron transport activity (Robinson et al., 1990). Phospholipase A₂ treated cytochrome c oxidase removes bound CL (Sedlak and Robinson, 1999). Depletion of CL from these enzymes result in a 60-70% decrease in activity, but enzyme function is restored after exogenous CL specifically binds to two high-affinity CL sites (Robinson et al., 1990). Due to CL's proton trapping ability, CL acts to shuttle protons from the oxidative phosphorylation complexes to the ATP synthase (Kates et al., 1993). Therefore restoration of full electron transport activity is exclusive for CL and no other phospholipid can reinstate this activity. Alteration in the content and the molecular species composition of CL will alter oxygen consumption in mammalian mitochondria (Yamaoka *et al.* 1990; Ohtsuka *et al.* 1993). CL interaction with mitochondrial proteins appears specific since substitution with other phospholipids does not fully reconstitute their activity. Indeed, it is suggested that CL may be the "glue" that holds the mitochondrial respiratory complex together (Zhang *et al.*, 2002). Thus, maintenance of the appropriate content of CL within the mitochondria is essential for proper mammalian cell function.

Cardiolipin and cell death

The role CL plays in apoptosis has been extensively reviewed (McMillin and Dowhan, 2002; Esposti, 2002). Studies have illustrated the role of CL in the function of proapoptotic proteins from the Bcl-2 family (Esposti et al.,

2003). These proteins such as Bid, Bax and Bak are responsible for initiating apoptosis via release of cytochrome c from the mitochondria. Apoptosis mediated by death receptors such as Fas will activate apical proapoptotic enzyme caspase 8 to cleave full length Bid protein at the C-terminus to form active truncated Bid (t-Bid), but it still remains associated with the N-terminal (n-Bid) (Esposti et al., 2001; Liu et al., 2005). The cleavage of Bid by caspase 8 exposes a hydrophobic area formed by helices H3 and H6 as the CL-binding domain, which is covered by helices H1 and H2 in full-length Bid (Liu et al., 2004). The dissociation of t-Bid from the N-terminal is necessary because n-Bid inhibits the membrane destabilizing effect of t-Bid required for apoptosis (Liu et al., 2005). The presence of CL or MLCL then attracts t-Bid to dissociate from n-Bid in the cytoplasm and move towards the mitochondrial outer membrane (Esposti et al., 2003; Liu et al., 2005). CL/MLCL molecules tend to aggregate together to form lipid domains in the outer membrane with specific properties that will prime the membrane for t-Bid and Bax/Bak activation (Hardwick and Polster, 2002). The specificity for CL is important for the relocation of t-Bid, except there is a decrease in CL during Fas-mediated apoptosis (Esposti et al., 2003). However, t-Bid has been shown to also bind to metabolites of CL such as MLCL, a byproduct of CL during apoptosis, therefore it can bind to t-Bid more readily. The increase in MLCL is perhaps due to apoptotic enhancement in CL remodeling, caused by activation of phospholipase C to deplete PC, which is the preferred acyl donor for MLCL reacylation by CL transacylase. Both t-Bid and CL/MLCL act as ligands for other

Bcl-2 proteins Bax/Bak, causing mitochondrial membrane permeabilization (MMP) (Zamzami and Kroemer, 2003). MMP occurs when activated Bax/Bak mediated by t-Bid-MLCL complex induce the oligomerization of Bax to insert itself into the mitochondrial membrane, forming channels (Zamzami and Kroemer, 2003; Liu et al., 2004). This causes a loss of barrier function of the outer mitochondrial membrane by increasing the permeability of the membrane to allow cytochrome c to be released from the intermembrane space to the cytoplasm. This then causes conformational changes to apoptosome, a caspase activation complex containing Apaf-1 and procaspase-9, to activate a cascade of caspase enzymes such as caspase 9 (Liu et al., 2004). Binding of CL to t-Bid disturb the function of mitochondrial respiratory proteins by competing for CL needed by these proteins. Bid may also be involved in transporting phospholipids such as CL from the mitochondria to the plasma membrane during Fas-mediated apoptosis (Sorice et al., 2004). Studies have revealed that addition of the proapoptotic factor $TNF\alpha$ to H9c2 cells stimulated mitochondrial phospholipase A_2 (PLA₂) activity towards CL (Xu et al. 1999). Loss of CL due to aging, nitric oxide, radiation, and ischemia can cause the release of cytochrome c, subsequently triggering apoptotic enzymes. The hydrophobic interaction and hydrogen bonding of one acyl chain from an acidic phospholipid such as CL to cytochrome c at its C-site cause a conformational change in cytochrome c, thus activating caspases in apoptosis (Tuominen et al., 2002; Jemmerson et al., 1999). The reduction of CL due to phospholipase also affects the function of proteins involved in energy metabolism, contributing to

apoptosis (Nakahara et al., 1992). Loss of CL also leads to the destabilization of the mitochondrial permeability transition pore as a result of events such as calcium overload, oxidative stress and loss of ATP, subsequently releasing of cytochrome c to initiate apoptosis (Crompton, 2000). Based upon these and several other studies, it appears that CL and its metabolism may play a central role in the pathway leading to cellular apoptosis.

Phospholipid scramblase-3

The asymmetric distribution of lipids within the membrane influences significant physiological functions, for instance cell viability, membrane fusion and shape, and cell-cell recognition (Tannert et al., 2003). Phospholipid scramblases (PLS) are membrane bound enzymes responsible for bi-directional movement of phospholipids (Bever et al. 1999). This transport or “scrambling” usually exhibits no specificity for the type of phospholipid or direction of the movement (Frasch et al., 2000). PLS activity has a significant role in apoptosis, where scrambling alters the phospholipid content of the membrane, such as exposure of phosphatidylserine to the outer leaflet of the plasma membrane, to prepare for recognition and removal of the cell by phagocytes. PLS proteins have short or no extracellular domains, whereas their intracellular domains are variable in composition and length (Yu et al., 2003). PLSs are transmembrane proteins that are highly conserved in the calcium-binding C-terminal domain, where calcium binding activates the protein (Yu et al., 2003). Phosphorylation of these proteins by protein kinases have also been shown to activate PLSs during apoptosis (Frasch et al., 2000). Four family

members have been identified. PLS1 is localized to the plasma membrane and is responsible for the translocation of phospholipids between inner and outer membranes, and PLS2 is localized predominantly in nucleus (Yu et al. 2003). PLS1 has also been shown to relocate to the nucleus by the importin α/β import pathway, and a receptor-mediated nuclear import signal for PLS1 in the nucleus has been identified, indicating a possible nuclear function (Ben-Efraim et al., 2004). A new member of the family, phospholipid scramblase-3 (PLS3), has recently been identified and is localized to the mitochondria (Figure 4). Although there is insufficient information regarding other physiological functions of PLS3 in mitochondria, PLS3 functions to transport CL from the inner to the outer mitochondrial membrane (Liu *et al.* 2003a,b). The enzyme is activated by phosphorylation from protein kinase C- δ (PKC- δ) and is dependent on the binding of Ca^{2+} due to a conserved calcium-binding motif. In addition, PLS3 is an important regulator of CL transport in apoptosis (Liu *et al.*, 2003a,b). It aids in the transport of CL from the inner mitochondrial membrane where it is synthesized to the outer mitochondrial membrane where it forms a complex with t-Bid to initiate Fas-mediated apoptosis. Overexpression of PLS3 elevated CL levels on the outer mitochondrial membrane and rendered U293 cells more susceptible to UV- and tBid-induced apoptosis whereas disruption of PLS3 caused cells to become less susceptible. Cells containing a disrupted PLS3 also showed slower growth rates,

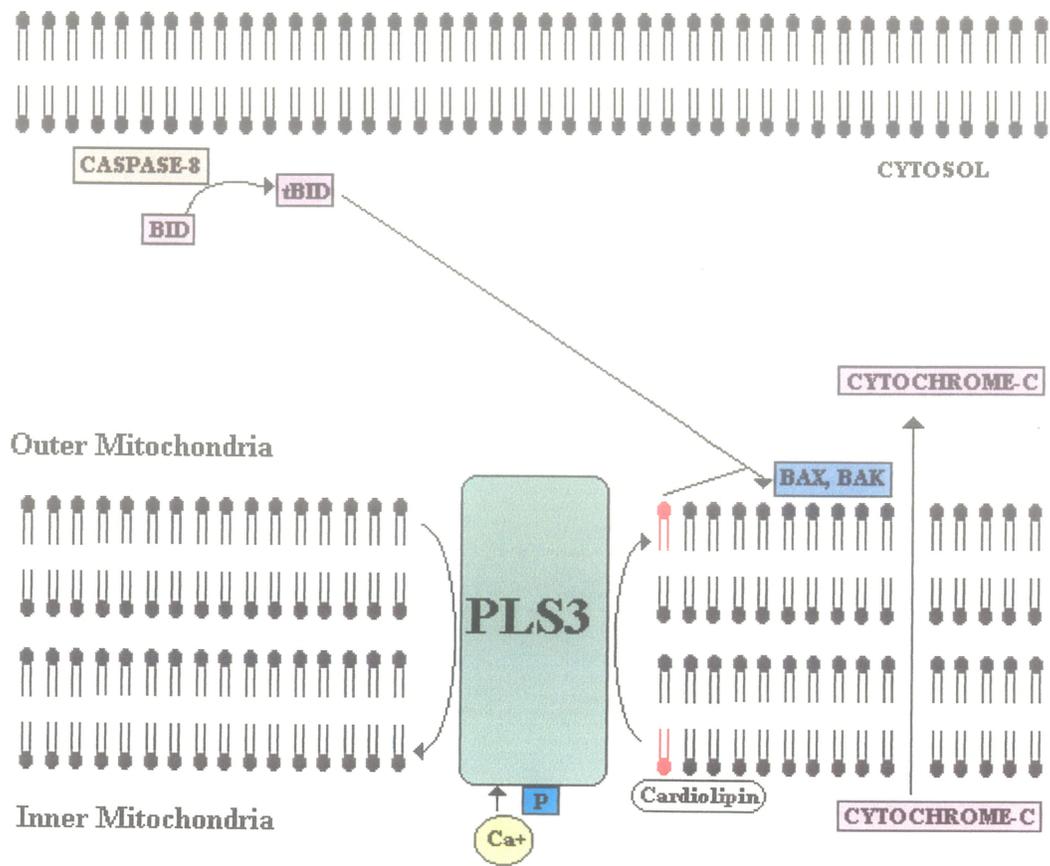


Figure 4: The role of cardiolipin and PLS3 in extrinsic pathway of apoptosis.

therefore PLS3 may play a role in cell growth. Studies also demonstrated a decrease in mitochondrial mass, transmembrane potential, intracellular ATP and mitochondrial respiration in PLS3-disrupted cells (Liu *et al.*, 2003a,b). PLS3 may also regulate t-Bid-mediated cytochrome c release in apoptosis, since PLS3 controls the trafficking of CL to the outer membrane. Cells with disrupted PLS3 display altered mitochondrial morphology, which are larger in size and numerous cristae packed together to give the mitochondria an abnormal shape. This is perhaps due to the overgrowing inner membrane and slower expansion of the outer membrane. Therefore, PLS3 may also regulate the development of the mitochondrial membrane by monitoring the distribution of phospholipids. Studies have also shown that PLS3 may play a role in lipid disorders, where a deletion in PLS3 resulted in insulin resistance, glucose intolerance, dyslipidemia and an increase in abdominal fat (Wiedmer *et al.*, 2004). It is unknown how the transport of CL from the inner membrane to the outer membrane affects the biosynthesis of CL. The purpose of this study was to examine if altering the levels of functional PLS3 regulates CL biosynthesis.

MATERIALS AND METHODS

Materials

[¹⁴C]Glycerol-3-phosphate, [³H]CTP and [³H]glycerol, [¹⁴C]linoleoyl-CoA, [¹⁴C]oleoyl-CoA, [¹⁴C]linoleic acid, and [³H]serine were obtained from either Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. [¹⁴C]PG was synthesized from [¹⁴C]glycerol-3-phosphate (Hatch 1994). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were products of Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. Hela cells were obtained from American Type Culture Collection. Hela cells overexpressing PLS3 (PLSCR3) or expressing a disrupted PLS3 (F258V) were obtained from Dr. Raymond Lee, Huntsman Cancer Institute, University of Utah (Figure 5). Western blot analysis system was used for protein expression studies and was obtained from Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England. Kodak X-OMAT film was obtained from Eastman Kodak Co., Rochester, NY., USA. Perkin-Elmer DNA Thermal Cycler was used for RT-PCR DNA sequencing through different temperatures. QIAGEN OneStep RT-PCR kit

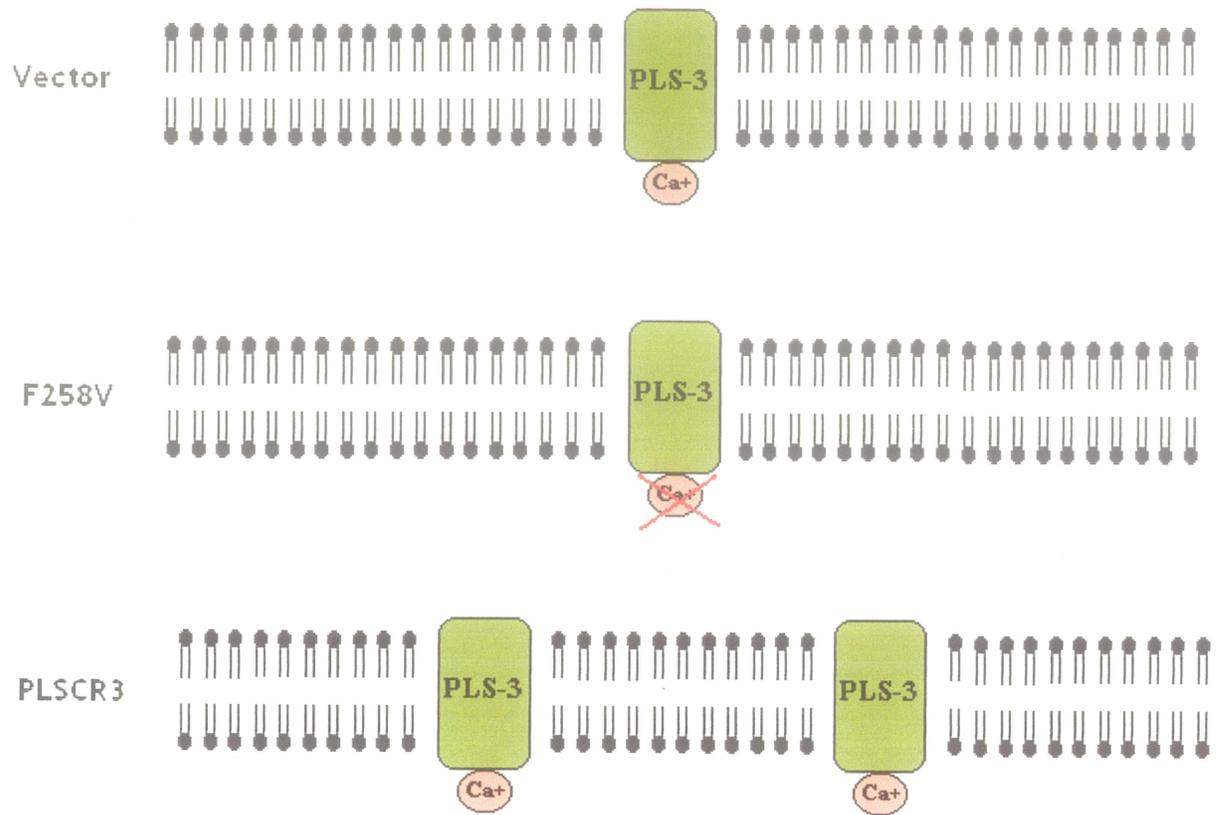


Figure 5: HeLa cell lines. Vector (control), F258V with a disrupted calcium-binding motif rendering it inactive, and PLSCR3 where the PLS3 protein is overexpressed.

were used for PCR studies, which included enzyme mix (containing Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase, and HotStarTaq® DNA Polymerase), RT-PCR buffer, Q-Solution, dNTP Mix and RNase-free water. PGPS, CLS, ER MLCL AT, mitochondrial MLCL AT and actin primers were used in PCR studies. All other biochemicals were certified ACS grade or better and obtained from Sigma Chemical Company, St. Louis, USA or Fisher Scientific, Winnipeg, Manitoba, Canada.

Culture, radiolabeling and harvesting of HeLa cells

HeLa cells were transfected with mutants where Phe²⁵⁸ was converted to val²⁵⁸ to disrupt the Ca²⁺-binding motif, the control (vector), or overexpressed (PLSCR3) were also transfected. HeLa vector, F258V and PLSCR3 cells were cultured and maintained in DMEM containing 10% fetal bovine serum until 70% confluence. Cells were then incubated for up to 8 h with DMEM in the absence or presence of 0.1 mM [1,3-³H]glycerol (10 μCi/10cm dish), 0.1 mM [1-¹⁴C]linoleic acid (bound to albumin 1:1 molar ratio) (1 μCi/dish), 0.1mM [³H]inositol (10 μCi/dish), or [³H]serine (10 μCi/dish). The medium was removed and the cells washed twice with cold Dulbecco's phosphate buffer saline and then harvested from the dish with 2 ml methanol: water (1:1 v/v). 2 Aliquots of 25μl were then taken from the samples for a protein assay and to measure total radioactivity. For lipid extraction, 0.5ml 0.9% sodium chloride and 2ml chloroform was added to each sample to form two separate phases, making the ratio in each sample 2:3:4 (methanol: water: chloroform). This was

followed by centrifugation at 2000xg rpm for 10 minutes and the upper phase removed. The sample was washed again with theoretical upper phase (methanol: 0.9% NaCl: water), centrifuged for 5 minutes, the upper phase removed again and bottom phase dried down with nitrogen. CL was separated from other phospholipids by two-dimensional thin-layer chromatography, where the phospholipids were revealed with iodine vapour and scraped off into 7ml vials with Ecolite scintillant. Radioactivity incorporated into CL and other phospholipids were determined by liquid scintillation using a Beckman scintillation counter.

Lowry's Protein Assay

Protein was determined by Lowry protein assay (Lowry et al., 1951). Standard curve was prepared using bovine serum albumin (1mg/ml) in volumes 0, 10, 20, 40 and 80 μ l. In each sample, 100 μ l 1% deoxycholic acid was added and vortexed. A 50:1 ratio mixture of 2% Na_2CO_3 in 0.1M NaOH and 0.5% CuSO_4 in 1% sodium citrate was prepared, and 2ml of this mixture was added to the samples, vortexed and allowed to sit for 10 minutes. A 1:1 H_2O : Folin reagent mixture was prepared and 200 μ l was added to each sample, vortexed and allowed to sit for 30 minutes. The absorbance of each sample was measured at 660nm (A_{660}).

Determination of in vitro enzyme activities

Hela vector, F258V and PLSCR3 cells were incubated for 3 days with DMEM containing 10% fetal bovine serum. The medium was changed once after 24 h. Subsequently, the cells were washed twice with cold phosphate buffered saline and harvested with 2 ml homogenisation buffer (10 mM Tris-HCL, pH 7.4, 0.25 M sucrose). The cells were homogenized with 30 strokes of a Dounce A homogeniser. The homogenate was centrifuged at 1,000 x g for 15 minutes and the resulting supernatant centrifuged at 10,000 x g for 20 min. The pellet was resuspended in 0.5 ml homogenization buffer and used to assay enzyme activities. Microsomal fractions from these cells were also isolated by taking the 10,000x g supernatant and centrifuging at 100,000 x g to obtain the pellet.

CTP: PA cytidyltransferase was assayed by a modification of the method of Carman and Kelley (1992). To a 16 mm x 100 mm test tube (all test tubes were treated with dimethyldichlorosilane, 20% in 1,1,1,-trichloroethane) was added, in this order, 0.05- 0.1 mg of mitochondrial protein and 0.145 M NaCl to a volume of 60 μ l, 10 μ l of 0.5 M Tris/maleate, pH 6.5 (0.5M Tris buffered with 1.0 M maleic acid solution), 10 μ l of 10 mM [5-³H]CTP (sp. radioactivity

12000 dpm/nmol), 10 μ l of 0.15 M Triton X-100, 5 mM PA (prepared by sonicating for 20 min a weighed sample of PA in the Triton solution in a silane-treated 16 mm x 100 mm tube). The reaction was started by addition of 10 μ l of 0.2 M MgCl₂·6H₂O. The mixture was incubated at 30 °C for 5 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid

extraction, 2 ml of chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of nitrogen and resuspended in 0.25 ml chloroform/methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first direction and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second direction. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting. Enzyme activity was linear with time up to at least 10 min with 0.1 mg of protein.

PGP synthase was assayed by a modification of the method of Carman and Belunis (1983). The incubation mixture contained enzyme (50-100 μ g) and 0.145 M NaCl added to a volume of 50 μ l, 10 μ l of 0.5 M Tris/HCl, pH 7.0, 10 μ l of β -mercaptoethanol (prepared fresh) and 10 μ l of 5.0 mM [U-¹⁴C]GP (sp. radioactivity 12500 dpm/nmol). The reaction was initiated by addition of 10 μ l of 100mM MgCl₂·6H₂O. The mixture was incubated at 30 °C for 10 min and terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid extraction, 2 ml of chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase

removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of nitrogen and resuspended in 0.25 ml chloroform/methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first direction and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second direction. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting. Enzyme activity was linear with time up to at least 15 min with 0.1 mg of protein.

CL synthase was assayed exactly as described by Schlame and Hostetler (1992), except that the assay contained 0.05-0.1 mg of protein, the pH of the assay was 8.5 and the samples were sonicated for 10 seconds in a Branson model 1200 sonicator before incubation. Incubation was at 37 °C for 60 min with [¹⁴C]PG (sp. radioactivity 45000 dpm/nmol) and terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid extraction, 2 ml of chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of nitrogen and

resuspended in 0.25 ml chloroform/methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first direction and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second direction. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting. CL synthase activity was linear for up to at least 70 min with 0.1 mg of protein. This assay could be performed with mitochondrial fraction that was previously frozen at -20 °C, without significant loss of activity.

For assay of MLCL AT activities, (50 µg protein) were incubated for 30 min at 25°C in 50 mM Tris-HCl, pH 9.0, 93 µM [1-¹⁴C]oleoyl-coenzyme A (6,700 dpm/nmol), or 33 µM [1-¹⁴C]linoleoyl-coenzyme A (68,700 dpm/nmol) at pH 8.0. Under these optimum assay conditions the reactions were linear up to 150µg protein and at least 40 min. The MLCL substrate in chloroform was dried under nitrogen and resuspended in double distilled water via sonication in a bath sonicator for 45 min prior to addition to the assay mixture. The temperature of the bath sonicator was maintained at 4°C by ice. The reaction was initiated by the addition of the radioactive acyl-coenzyme A substrate and terminated by the addition of 3 ml of chloroform-methanol 2:1 (v/v). To facilitate phase separation, 0.8 ml of 0.9% KCl was added. The aqueous phase was removed and the organic phase was washed with 2 ml of chloroform-methanol-0.9% NaCl 3:48:47 (v/v/v). The resulting organic fraction was dried

under nitrogen and resuspended in 25 μ l of chloroform-methanol 2:1 (v/v). A 20- μ l aliquot of the resuspended organic phase was placed on a thin-layer plate and CL was separated from other phospholipids in a solvent system containing chloroform-hexane-methanol-acetic acid 50:30:10:5 (v/v/v/v). Separation of CL from other lipids was confirmed using a two-dimensional thin-layer chromatography system described previously. The silica gel corresponding to CL was removed and placed in a plastic scintillation vial and 5 ml of scintillant added. Radioactivity incorporated into CL was examined approximately 24 h later using a liquid scintillation counter. MLCL AT activity was taken as radioactivity incorporated into CL in the presence of the MLCL substrate minus radioactivity incorporated into CL in the absence of the MLCL substrate.

Endoplasmic reticulum (ER) MLCL AT activity was determined exactly as mitochondrial MLCL AT, except using a 5 μ g microsomal fraction. PLA2 activity was determined using 100 μ g mitochondrial fraction with water to a volume of 80 μ l, 10 μ l of 350mM Tris-HCL, 10 μ l of [3 H]phosphatidylcholine (100,000 dpm/mg), and 10 μ l of 200mM Ca $^{+}$. 10 μ l was then removed to measure total radioactivity. Incubation was started by the addition of [3 H]phosphatidylcholine at 37°C for 60 min and terminated by the addition of 3 ml of chloroform-methanol 2:1 (v/v). To facilitate phase separation, 0.8 ml of 0.9% KCl was added. The aqueous phase was removed and the organic phase was washed with 2 ml of chloroform-methanol-0.9% NaCl 3:48:47 (v/v/v). The resulting organic fraction was dried under nitrogen and resuspended in 25 μ l of chloroform-methanol 2:1 (v/v). A 20- μ l aliquot of the resuspended organic

phase was placed on a thin-layer plate and CL was separated from other phospholipids in a solvent system containing chloroform-hexane-methanol-acetic acid 50:30:10:5 (v/v/v/v). Separation of CL from other lipids was confirmed using a two-dimensional thin-layer chromatography system. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting. Lysophosphatidylglycerol acyltransferase (LPG AT) activity was determined by adding 100 µg of mitochondrial fraction in water to a volume of 60 µl, 10 µl of 50 µM LPG, 10 µl of 50 µM linoleoyl-CoA and 10 µl Tris-HCl (pH 8.0). The reaction was initiated by the addition of 10 µl of [¹⁴C]linoleoyl-CoA (222,000 dpm/nmol), incubation at 37°C for 60 min, and terminated by adding 1.5 ml of water and 3ml chloroform:methanol (2:1 v/v). For phase separation, 0.8 ml of 0.9% KCl was added. The aqueous phase was removed and the organic phase was washed with 2 ml of chloroform-methanol-0.9% NaCl 3:48:47 (v/v/v). The resulting organic fraction was dried under nitrogen and resuspended in 25µl of chloroform-methanol 2:1 (v/v). A 20-µl aliquot of the resuspended organic phase was placed on a thin-layer plate and CL was separated from other phospholipids in a solvent system containing chloroform-hexane-methanol-acetic acid 50:30:10:5 (v/v/v/v). Separation of CL from other lipids was confirmed using a two-dimensional thin-layer chromatography system described previously. The silica gel corresponding to CL was removed and placed in a plastic scintillation vial and 5 ml of scintillant added. Radioactivity incorporated into CL was examined approximately 24 h

later using a liquid scintillation counter. Lysophosphatidylethanolamine acyltransferase (LPE AT) activity was determined exactly as LPG AT, except 50 μM of LPE was used as substrate.

RT-PCR analysis

The cDNA for MLCL AT was amplified with specific primers synthesized by Invitrogen™ Life Technologies. Total RNA from vector control, PLSCR3 and F258V Hela cells was isolated using the TRIZOL Reagent according to the manufacturers instructions. The treated cells were then pipetted into a small capped tube, 0.2ml of chloroform was added, and each sample was shaken vigorously and centrifuged at 14000xg for 15min. The supernatant was removed to another tube and 0.5ml of isopropyl alcohol was added, causing the RNA to precipitate. This was centrifuged again at 14000xg for 10min to obtain the RNA pellet, which was then washed with 75% ethanol and centrifuged for 5min. The ethanol was then removed and the RNA pellet was resuspended in autoclaved, double-distilled water and quantitated by absorbance at 260 nm using the 260:280 nm ratio as an index of purity. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis of the isolated RNA sample. The first strand cDNA from 1 μg total RNA was synthesized by employing 150 U of moloney murine leukemia virus reverse transcriptase (RT), 25 pmol of random heamer primer, 20 U of ribonuclease inhibitor, 1 mM dithiothreitol, and 10 pmol each of the four deoxynucleotides, in a total volume of 15 μl . The reaction mixture was incubated at 37°C for 1 h and

terminated by boiling the sample at 95°C for 5 min. An aliquot of the resultant cDNA preparation was used directly for each amplification reaction. The primers for human PGPS (Genbank NO: BC025951): Forward 5'-GCT CAT GAA GGG GCA GAT AA-3'; Reverse 5'-CTG CAG CTG CAG GGA CAC AT-3'. PCR product length is 508 bp. The primers for CLS: Forward 5'-TTT GTT GGA TGG ATT TAT TGC TC-3'; Reverse 5'-TGT TCG TGG TGT TGG AAG AG-3'. PCR product length is 226 bp. Primers for ER MLCL AT: Forward 5' -AAC ACG GTC TTC GAT GCC AAG CGC CTC-3'; Reverse 5' -GGT CAA ACA CAA GGA TGT TCT TCT CTC-3'. PCR product length is 423 bp. The primers for human β -actin: Forward 5'-GTG GGG CGC CCC AGG CAC CA-3'; Reverse 5'-CTC CTT AAT GTC ACG CAC GAT TTG-3'. PCR product length is 540 bp. Polymerase chain reaction (PCR) was performed in 20 μ l reaction mixtures containing 8 pmol of primer, 8 pmol of each dNTP, and 0.4 U of *Taq* DNA polymerase. The mixture was overlaid with 30 μ l of mineral oil to prevent evaporation and was incubated in a Perkin-Elmer DNA Thermal Cycler under the following conditions: denaturation, 94°C, 30 sec; annealing, 58°C, 30 sec; extension: 72°C, 1.5 min; repeat for 26 cycles. The amplified RT-PCR product was analyzed by agarose gel (1.5%) electrophoresis in 1 X TAE buffer (40 mM Tris acetate, 2 mM sodium EDTA) and visualized by staining with 0.5 μ g ethidium bromide. PCR conditions for PGPS and MLCL AT: denaturation 94°C, 1 min; annealing 55°C, 1 min; extension 72°C, 30 s; repeat for 25 cycles. PCR conditions for CLS: denaturation 94°C, 50 s; annealing 60°C, 50 s; extension 72°C, 2 min; repeat

for 35 cycles. The amplified RT-PCR product was analyzed by agarose gel (1.2%) electrophoresis as described above.

An increase in the level of a specific mRNA is caused by either an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of these two processes. Hence, mRNA stability assays were conducted using actinomycin D as an inhibitor of RNA synthesis. The mRNA levels for MLCL AT, PGPS and β -actin were determined by RT-PCR at 4 h intervals following the actinomycin-treatment. No apparent changes in mRNA degradation were observed within a 24 h period, indicating PLS3 disruption did not cause any change in the degradation of the mRNA of each enzyme. The relative intensities of the bands were analyzed by scanning the film, and subsequently determined by Scion Image software.

Electrophoresis and Western blot analysis

Hela cells were incubated for 3 days with DMEM containing 10% fetal bovine serum. The medium was changed once after 24 h. The cells were harvested, homogenized and cellular fractions prepared as described above. A 25 μ g aliquot of mitochondrial samples and SDS/7.5% PAGE containing molecular weight standards was added to a BioRad Mini-Protean[®] II Dual Slab Cell electrophoresis unit. Proteins were transferred from the gel onto PVDF membranes by incubation for 90 min at 15 volts using a BioRad Trans-Blot SD Semi-Dry Transfer Cell. Expression of MLCL AT was examined by incubating the PVDF membrane with the MLCL AT antibody (Taylor and Hatch 2003) (1:1000

dilution) dissolved in Tris-buffered saline containing 0.1% Tween-20 and 2% skim milk for overnight at 4°C. Subsequently, the membrane was washed and incubated with peroxidase labeled anti-rabbit secondary antibody (1:5000) for 5-30 min at room temperature. Protein bands in the membrane were visualized by enhanced chemiluminescence.

Other determinations

ANOVA statistical analysis was used for determination of statistical significance. The level of significance was defined as $p < 0.05$.

RESULTS

Radioactivity incorporation

To determine if disruption or overexpression of PLS3 has an effect on the CL *de novo* biosynthesis, HeLa cell lines vector (control), F258V (PLS-disrupted) and PLSCR3 (PLS-overexpressed) were incubated for 8 hours with [³H]glycerol and the radioactivity incorporated into CL and PG of the CDP-DG CL biosynthesis pathway determined as described in Materials and Methods. Incorporation of radioactivity into CL was 174 dpm/mg, 313 dpm/mg, and 369 dpm/mg for vector, F258V and PLSCR3, respectively (Table 1). When compared to the control cells, [³H]glycerol incorporation into CL was 2.1-fold and 1.8-fold higher in F258V and PLSCR3, respectively. Incorporation of radioactivity into PG was 300 dpm/mg, 468dpm/mg, and 369 dpm/mg for vector, F258V and PLSCR3 cells, respectively (Table 1). When compared to vector cells, only a minor elevation in radioactivity incorporation into PG was observed. Radioactivity incorporation into PA and CDG-DG, other lipids in the CDP-DG pathway, were at background levels (<30 dpm/mg protein). The total [³H]glycerol uptake into the cells after 8 hour incubation was 2.1×10^5 dpm/mg in vector cells, 2.1×10^5 dpm/mg in F258V cells, and 2.2×10^5 dpm/mg in PLSCR3 cells. Thus there was no difference in uptake of [³H]glycerol observed between the cell lines. Therefore, disruption or overexpression of functional PLS3 protein increases *de novo* CL biosynthesis from glycerol.

Phospholipid	Control	F258V dpm/mg protein	PLSCR3
Cardiolipin	174 ± 11	313 ± 46	369 ± 54
Phosphatidylglycerol	300 ± 45	368 ± 63	378 ± 52

Table 1: Synthesis of phospholipids of the CDP-DG pathway from [³H]glycerol in HeLa cell lines containing a disrupted PLS3 (F258V) or overexpressing PLS3 (PLSCR3). HeLa vector, F258V and PLSCR3 were incubated with 0.1 mM [³H]glycerol for 8 h and the radioactivity incorporated into phospholipids determined as described in Materials and Methods. Values represent the mean of two separate experiments.

To study the effect of altered levels of functional PLS3 protein on non-mitochondrial phospholipid biosynthesis, HeLa vector, F258V and PLSCR3 cells were incubated with [³H]inositol for 8 hours and radioactivity incorporated into phosphatidylinositol (PI) determined. Incorporation of [³H]inositol into PI was 5442 ± 782 dpm/mg protein, 4780 ± 674 dpm/mg, and 6421 ± 575 dpm/mg in vector, F258V and PLSCR3 cells, respectively (Figure 6). The total [³H]inositol uptake was 1.9×10^5 dpm/mg, 1.8×10^5 dpm/mg and 1.9×10^5 dpm/mg for vector, F258V and PLSCR3 cells respectively. Thus, there were no difference in [³H]inositol uptake into PI between cells.

To study the effect of altered levels of functional PLS3 on a different phospholipid synthesized within the mitochondria, biosynthesis of phosphatidylethanolamine (PE) from phosphatidylserine (PS) was determined. PS synthesized in ER is rapidly transported to mitochondria where it is decarboxylated to form PE. The three HeLa cell lines were incubated with [³H]serine and incorporation of radioactivity into PS and PE determined. Incorporation of radioactivity into PS was 11270 dpm/mg in vector cells, 12134 dpm/mg in F258V cells, and 12117 dpm/mg in PLSCR3 cells (Table 2). Incorporation of [³H]serine into PE in each cell line was 17291 dpm/mg, 17633 dpm/mg and 18475 dpm/mg for vector, F258V and PLSCR3 cells, respectively. The total [³H]serine uptake into vector, F258V and PLSCR3 was 258.8×10^4 dpm/mg, 263.1×10^4 dpm/mg and 225.8×10^4 dpm/mg, respectively.

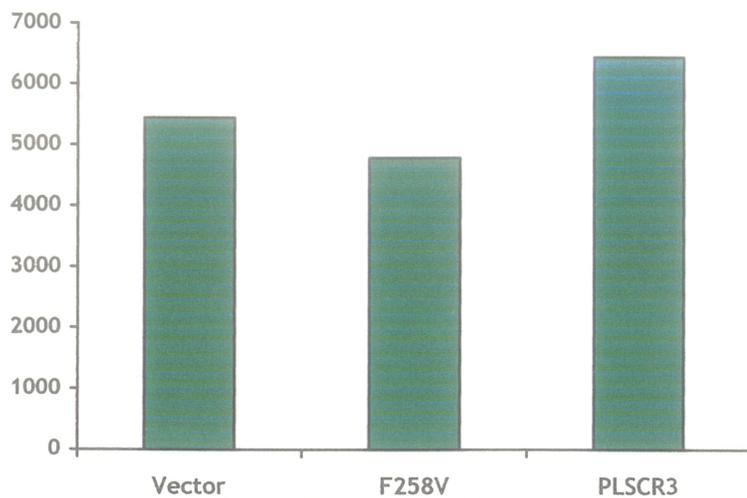


Figure 6: [³H]Inositol incorporation into PI in HeLa cells with disrupted PLS3 and overexpressed PLS3. HeLa cells vector, F258V and PLSCR3 were incubated in 0.1mM [³H]Inositol for 8 hours and the radioactivity incorporated into PI determined as described in Materials and Methods. Values (expressed as dpm/mg protein) represent the mean of two separate experiments.

	Control	F258V	PLSCR3
		dpm x10 ⁴ /mg protein	
Total uptake of [³ H]serine	258.8	263.1	225.8
		dpm/mg protein	
Phosphatidylserine	11,270 ± 167	12,134 ± 423	12,117±1602
Phosphatidylethanolamine	17,291 ± 471	17633 ± 218	18475 ± 1142

Table 2: Synthesis of phospholipids from [³H]serine in HeLa cell lines with disrupted PLS3 or overexpressed PLS3. HeLa vector, F258V and PLSCR3 were incubated with [³H]serine for 8 h and the radioactivity incorporated into phospholipids determined as described in Materials and Methods. Values represent the mean of two separate experiments.

Therefore alteration in functional of PLS3 protein had no effect on uptake of [³H]serine, its incorporation into PS or the biosynthesis of PE within the mitochondria.

To study if disruption or overexpression of PLS3 would influence CL remodeling, HeLa vector, F258V and PLSCR3 cells were incubated with [¹⁴C]linoleic acid for 8 hours and the radioactivity incorporated into CL and PG determined as described in Methods and Materials. Radioactivity into PG was 9717 ± 1599 dpm/mg, 5712 ± 515 dpm/mg, and 3395 ± 443 dpm/mg for vector, F258V and PLSCR3 respectively (Figure 7). Hence, disruption or overexpression of PLS3 in HeLa cells resulted in a 41% or 65% decrease in [¹⁴C]linoleic acid incorporation into PG, respectively, compared to vector control cells. Radioactivity incorporated into CL in HeLa vector, F258V and PLSCR3 cells was 28671 dpm/mg, 15847 dpm/mg, and 14036 dpm/mg, respectively. When compared to vector cells, disruption or overexpression of PLS3 protein resulted in a 45% and 51% decrease in [¹⁴C]linoleic acid incorporation into CL, respectively. Thus disruption or overexpression of PLS3 decreases CL remodeling.

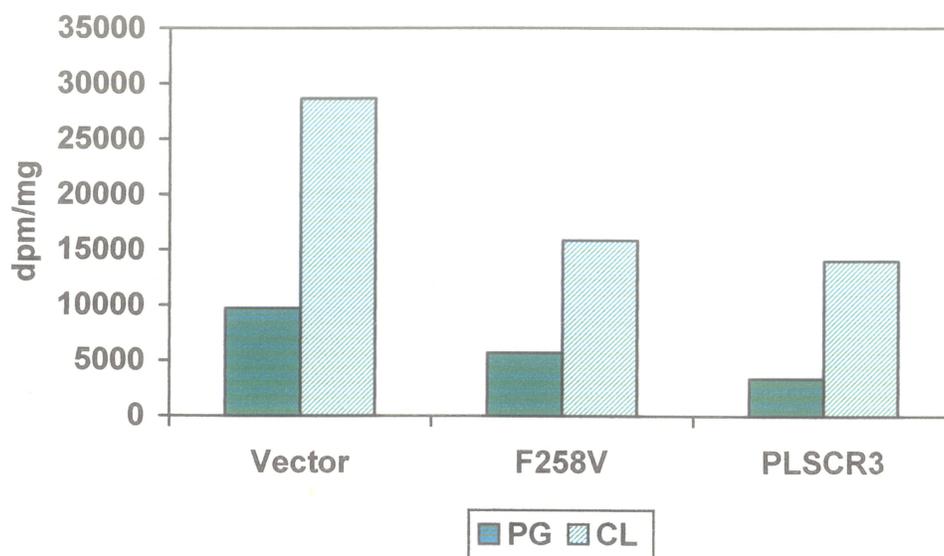


Figure 7: [¹⁴C]linoleic acid incorporation into PG and CL in HeLa cell lines overexpressing PLSCR3 or containing a disrupted PLS3. HeLa control cells or HeLa cells overexpressing PLS3 (PLSCR3) or HeLa cells containing a disrupted PLS3 (F258V) were incubated with 0.1 mM [¹⁴C]linoleic acid for 8 h and the radioactivity incorporated into PG and CL determined as described in Materials and Methods. Values (expressed as dpm/mg protein) represent the mean of two separate experiments.

Enzyme activities of the CDP-DG pathway and CL remodeling enzymes

The alteration in radioactivity incorporated into CL could be due to a change in the activity of CL synthase. Thus, activities of the enzymes involved in the biosynthesis and remodeling of CL were examined (Table 3). CDS activities (PA to CDP-DG) in Hela vector, F258V and PLSCR3 were 20, 18, and 21 pmol/min/mg in vector, F258V and PLSCR3, respectively. PGP synthase activity (CDP-DG and glycerol-3-phosphate to PGP) were 448, 494, and 495 pmol/min/mg protein in vector, F258V and PLSCR3 cells respectively. Thus no apparent differences in CDS or PGP synthase activities were observed between the different cell lines. In contrast, CL synthase activities (CDP-DG and PG to CL) were 1.4, 3.7, and 2.3 pmol/min/mg protein in vector, F258V and PLSCR3 cells, respectively. Thus CL synthase activity was elevated 2.6-fold in F258V and 1.6-fold in PLSCR3 cells compared to vector control. The increase in CL synthase activities in the PLS3-disrupted and PLS3-overexpressing cells coincided with the increase in [³H]glycerol incorporation into CL (Table 1).

To study whether altered levels of functional PLS3 affected the remodeling of CL, phospholipase A₂ (PLA₂) and mitochondrial MLCL AT activities were determined. PLA₂ activities (deacylation of CL to MLCL) were 191 pmol/min/mg in vector, 180 pmol/min/mg in F258V, and 200 pmol/min/mg in PLSCR3 cells. Thus, there were no differences between the cells, indicating altering levels of PLS3 has no effect on the deacylation of CL. However, MLCL AT activities (reacylation of MLCL to form remodeled CL) were 472, 329, and

Enzymes	Control	F258V	PLSCR3
	pmol/min/mg protein		
CDS	20 ± 4	18 ± 4	21 ± 12
PGPS	448 ± 16	494 ± 64	495 ± 42
CLS	1.4 ± 0.2	3.7 ± 0.8*	2.3 ± 0.5*
PLA ₂	191 ± 34	200 ± 25	180 ± 40
MLCL AT (Lin)	472 ± 38	329 ± 3*	403 ± 16*
MLCL AT (Ol)	181 ± 38	80 ± 3*	95 ± 16*
MLCL AT (ER)	52 ± 8	85 ± 6*	94 ± 5*
LPG AT	4.9 ± 1.8	4.7 ± 0.4	4.6 ± 0.9
LPE AT	6.2 ± 1.1	4.6 ± 0.34	3.7 ± 1.0

Table 3: Activities of the de novo CL biosynthesis, remodeling enzymes, LPG AT and LPE AT in HeLa cells possessing a disrupted PLS3 or overexpressing PLS3. Enzyme activities in mitochondrial fractions prepared from vector, F258V and PLSCR3 cells were determined as described in Materials and Methods. Values represent the mean standard deviation of three dishes *p<0.05.

403 pmol/min/mg in vector, F258V and PLSCR3 cells, respectively, when linoleoyl-CoA was used as a substrate. With linoleoyl-CoA as a substrate a modest 30% or 15% decrease in MLCL AT activities were observed in the PLS3 disrupted or overexpressed cells, respectively, when compared to control. MLCL AT activity was then determined using [¹⁴C]oleoyl-CoA as a substrate. MLCL AT activities were 180 pmol/min/mg, 80 pmol/min/mg, and 95 pmol/min/mg for vector, F258V and PLSCR3 cells, respectively. Thus, MLCL AT activities were reduced 55% or 48% in F258V or PLSCR3 cells, respectively, compared to vector control.

ER MLCL AT activities were then determined using [¹⁴C]linoleoyl-CoA as substrate. ER MLCL AT activities were 52, 85, and 94 pmol/min/mg in vector, F258V and PLSCR3 cells, respectively. Thus, ER MLCL AT activity was increased 63% in F258V cells and 81% in PLSCR3 cell compared to control. To study if the activity of other acyltransferases were affected by alterations in functional PLS3, LPG AT and LPE AT activities were determined. LPG AT activities (for reacylation of PG) were 4.89, 4.71, and 4.61 pmol/min/mg for vector, F258V and PLSCR3 cells, respectively, exhibiting no differences. LPE AT activities (for the reacylation of PE) were 6.22, 4.62, and 3.71 pmol/min/mg for vector, F258V and PLSCR3 cells. Thus, LPE AT were reduced 26% or 40% decrease in F258V or PLSCR3 cells, respectively, compared to vector control. CL transacylase activity was not determined since the activity of the enzyme is low (reviewed Hauff and Hatch, 2006) and could not be detected in Hela cells.

RT-PCR and Western Blot Analysis

RT-PCR experiments were performed to determine if altered levels of functional PLS3 affected the relative RNA levels of CL biosynthesis and remodeling enzymes. Total RNA was isolated from vector, F258V and PLSCR3 and the relative expression of PGP synthase, CL synthase, ER and mitochondrial MLCL AT were determined by RT-PCR as described in Methods and Materials. The relative expression of PGP synthase was unaltered by disruption or overexpression of PLS3 compared to control (Figure 8 A & B). When compared to β -actin no significant change in PGP synthase expression between vector, F258V or PLSCR3 cells was observed (Figure 8C). However, there was a 78% and 52% increase in the relative expression of CL synthase in F258V and PLSCR3 cells, respectively, compared to control (Figure 9). The relative expression of mitochondrial MLCL AT was unaltered between vector, F258V and PLSCR3 cells (Figure 10). In contrast, there was a 38% and 84% increase in the relative expression of ER MLCL AT in F258V and PLSCR3 cells, respectively, compared to vector cells (Figure 11). This corresponded to the observed increases in enzyme activity in these cells (Table 3).

Western blot analysis of mitochondrial MLCL AT protein expression was performed in mitochondrial fractions using anti-MLCL AT antibody. There were no observable differences in the protein level of MLCL AT between vector, F258V and PLSCR3 cells (Figure 12). The lack of change in mitochondrial MLCL AT protein levels in the PLS3 disrupted and PLS3 overexpressing cells when

compared with controls coincided with the unaltered mRNA levels of the mitochondrial MLCL AT (Figure 10).

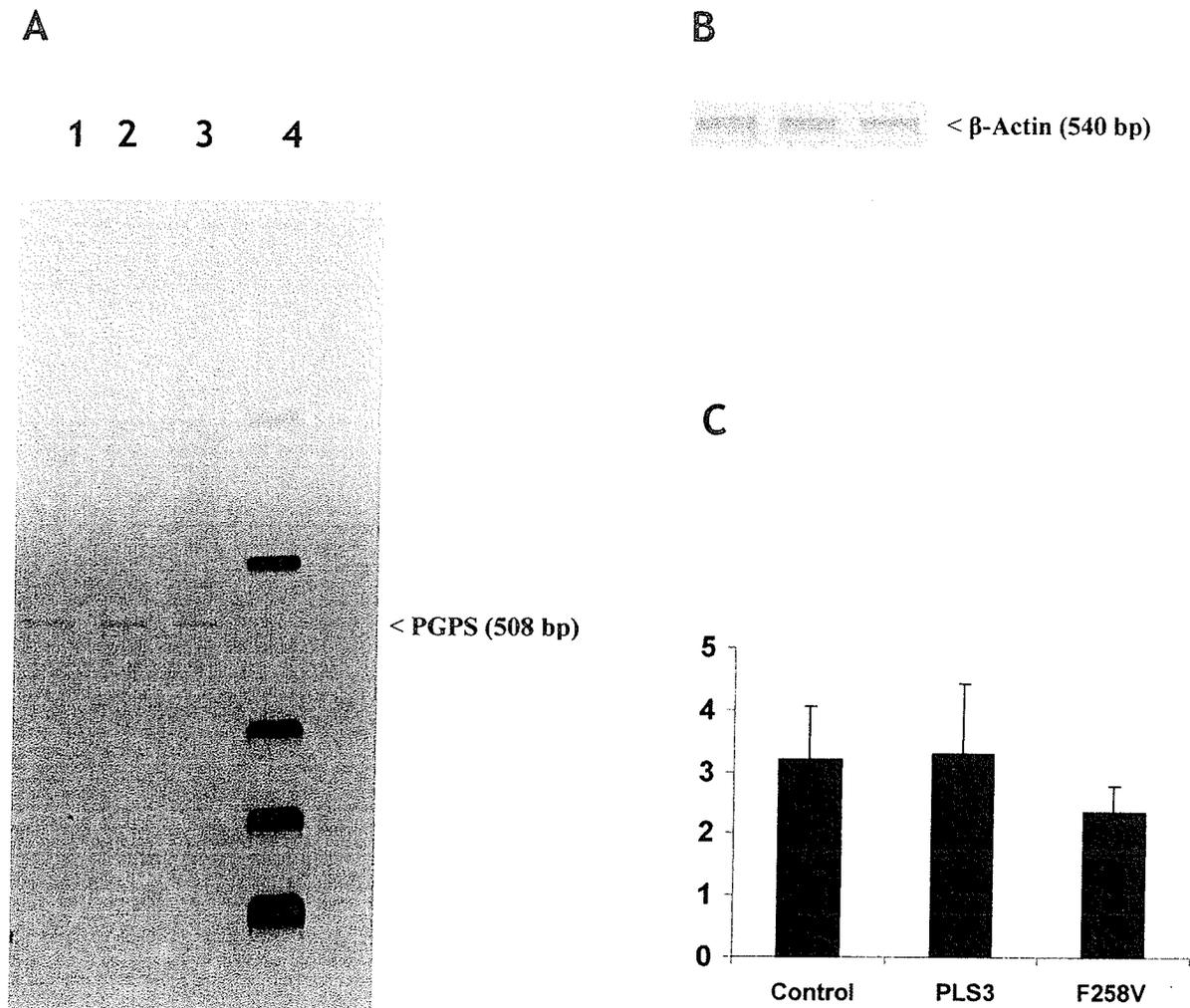


Figure 8: Expression of PGPS mRNA in HeLa cell lines overexpressing PLS3 or containing a disrupted PLS3. Total RNA was isolated from HeLa cells (control) or HeLa cells overexpressing PLS3 (PLSCR3) or HeLa cells containing a disrupted PLS3 (F258V) and the relative levels of PGPS mRNA determined by RT-PCR as described in Materials and Methods. A and B: Lane 1, control; Lane 2, PLSCR3, Lane 3, F258V, Lane 4, molecular mass markers. C: Ratio of the relative expression of PGPS to β -actin. The relative expression of the bands were determined as described in Materials and Methods.

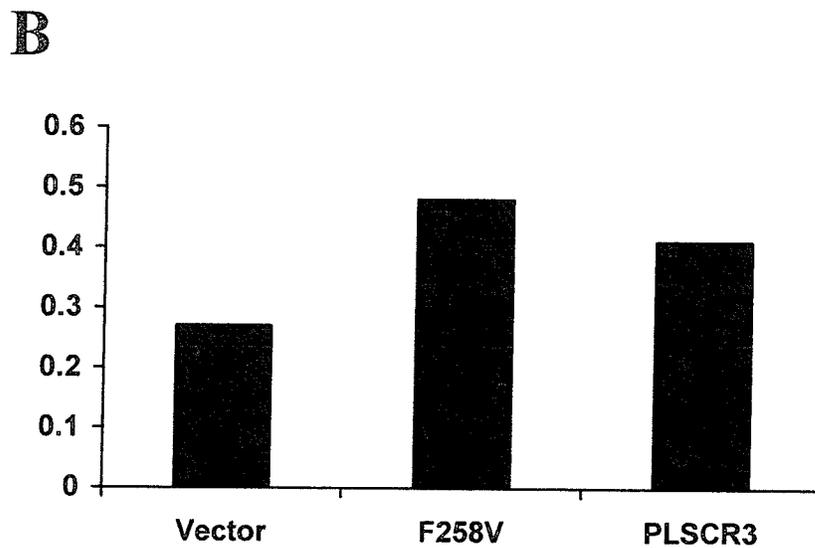
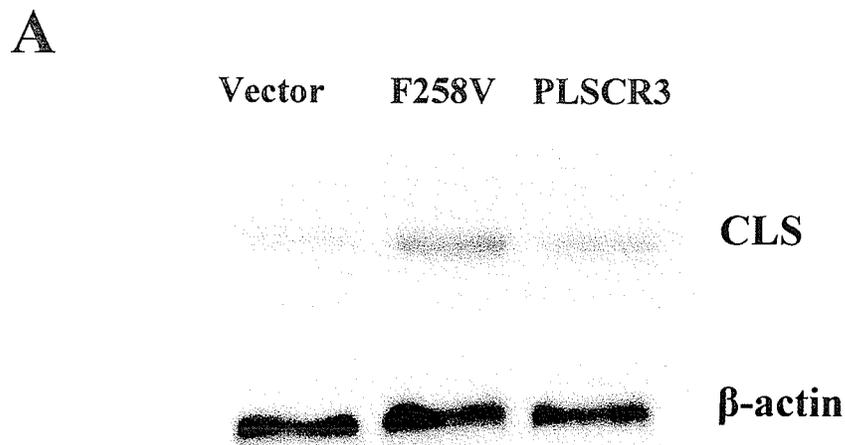


Figure 9: RT-PCR analysis of CL synthase RNA in HeLa cell lines overexpressing PLS3 or containing a disrupted PLS3. A: Cellular fractions were prepared and RT-PCR analysis of CL synthase in vector control HeLa cells (Lane 1) or F258V cells (Lane 2) or PLSCR3 cells (Lane 3) performed as described in Materials and Methods. B: Ratio of relative expression of CLS to β -actin.

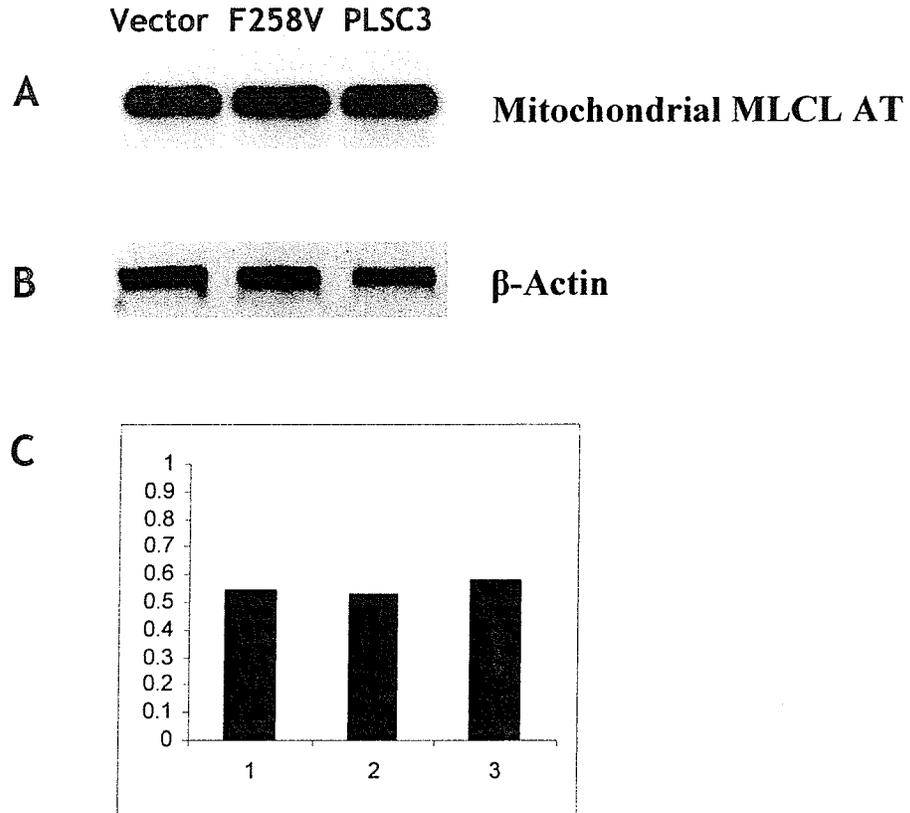


Figure 10: RT-PCR analysis of mitochondrial MLCL AT RNA in HeLa cell lines overexpressing PLS3 or containing a disrupted PLS3. A & B: Cellular fractions were prepared and RT-PCR analysis of mitochondrial MLCL AT in control HeLa cells (Lane 1) or F258V cells (Lane 2) or PLSCR3 cells (Lane 3) performed as described in Materials and Methods. C: Ratio of relative expression of mitochondrial MLCL AT to β -actin.



Figure 12: Western blot analysis of mitochondrial MLCL AT. Band 1 (standard), band 2 and 3 (vector), band 4 and 5 (F258V), band 6 and 7 (PLSCR3). Western blot performed as described in Materials and Methods.

DISCUSSION

The objective of this study was to investigate if different functional levels of phospholipid scramblase-3, a protein responsible for the transfer of CL between the inner mitochondrial membrane to the outer membrane (Liu et al., 2003 a,b), could influence CL biosynthesis and remodeling. Studies with vector control, F258V and PLSCR3 cells showed that various radioactive lipid precursors could be incorporated into products of the CDP-DG pathway including CL in Hela cells. Incorporation of [³H]glycerol into CL was increased in the F258V and PLSCR3 cells, indicating that CL biosynthesis from the precursor glycerol was increased when PLS3 was either disrupted or overexpressed. This coincided with the results of the CL synthase studies, where an increase in CL synthase activity and relative levels of CL synthase mRNA were observed when PLS3 was either disrupted or overexpressed. There were more modest alterations in [³H]glycerol incorporation into PG in F258V and PLSCR3 cells compared to control. RT-PCR analysis indicated there were no changes in mRNA levels of PGP synthase in F258V or PLSCR3 cells compared to control. Therefore, alteration in the levels of functional PLS3 do not likely affect PGP synthase expression. In support of this was the observation that PGP synthase enzyme activities were unaltered in F258V and PLSCR3 cells compared to control. Thus, the increase in the synthesis of CL in these cells was likely due to increase in CL synthase activity producing more CL. Disrupted or overexpressed PLS3 may alter the level and distribution of phospholipids

such as CL among the inner and outer mitochondrial membrane (Liu et al., 2003a). In the case of cells containing disrupted PLS3, there was an accumulation of CL in the inner mitochondrial membrane and a deficiency in the outer mitochondrial membrane (Liu et al., 2003a). In our study, this deficiency might send a positive feedback signal to increase the biosynthesis of CL in order to restore the appropriate level of CL to the outer membrane where it is needed for cellular functions such as apoptosis. Indeed, in F258V cells a decrease in the ability to undergo UV- and t-Bid-induced apoptosis was observed (Liu et al., 2003 a,b). In contrast in PLSCR3 cells overexpressing PLS3, there was an accumulation of CL on the outer membrane and a deficiency of CL in the inner membrane (Liu et al., 2003a). The observed increase in CL synthesis in PLSCR3 cells could act to replace the lowered level of CL on the inner membrane. As mentioned in the Introduction, the appropriate CL level in the inner mitochondrial membrane is essential for the role of CL in the mitochondria, specifically in energy metabolism (reviewed in Hatch 2004). Loss of CL leads to the decrease in activity of many enzymes, such as ATP-ADP carrier and respiratory complexes. Perhaps the loss of CL in the inner membrane due to overexpressed PLS had somehow distressed these complexes, a response not yet studied, leading to increased CL biosynthesis.

Incorporation of [³H]serine into PS and mitochondrial PE, and incorporation of [³H]inositol into PI were unaltered in F258V and PLSCR3 cells compared to control. Therefore, altered levels of functional PLS3 did not affect the biosynthesis of another mitochondrial phospholipid (PE) nor a non-

mitochondrial phospholipid (PI). Consequently, the cell may somehow detect only modified CL levels in mitochondrial membranes and the increase in CL biosynthesis was a compensatory mechanism to restore the appropriate levels of CL for proper cell function.

PLA₂ activity was unaltered in F258V and PLSCR3 cells compared to control, indicating that deacylation of CL was not affected by altering functional levels of PLS3. Radiolabeling with [¹⁴C]linoleic acid was subsequently performed to examine if altering the functional levels of PLS3 affected the incorporation of linoleic acid into CL during the reacylation of MLCL. The results indicated that [¹⁴C]linoleic acid incorporation into CL in both F258V and PLSCR3 cells was reduced when compared to controls, indicating a decrease in the reacylation of MLCL to CL. This was further supported by the observed decrease in mitochondrial MLCL AT activity in F258V and PLSCR3 cells, suggesting that the decrease in reacylation of MLCL to CL was due to decreased activity of the mitochondrial form of the enzyme. RT-PCR analysis indicated that there was no alteration in mRNA levels of mitochondrial MLCL AT in F258V or PLSCR3 cells compared to control. In addition, western blot analysis of mitochondrial MLCL AT revealed that there were no changes in protein expression of the enzyme in these cells. Thus, the observed decrease in mitochondrial MLCL AT activity in F258V and PLSCR3 cells was not due to decrease in the levels of its mRNA or protein expression. It is likely that the mitochondrial MLCL AT machinery is extremely sensitive to changes in CL distribution within the mitochondria and that alteration of CL localization may

alter the mitochondrial membrane such that the presentation of substrates to the enzyme is attenuated. In support of this was the observed reduction in LPG AT and LPE AT in cells expressing either an elevated or disrupted PLS3. In contrast, ER MLCL AT activity was increased in cells expressing either a disrupted (F258V) or elevated PLS3 (PLSCR3) and this coincided with an increase in ER MLCL AT mRNA levels as revealed by RT-PCR analysis. The expression of this enzyme was perhaps elevated in response to the decreased activity of the mitochondrial MLCL AT which would lead to a reduced level of linoleic acid incorporation into CL. Alternatively, the increase in ER MLCL AT could be linked to the elevated CL synthase activity observed in these cells (Taylor et al., 2002). The fact that linoleic acid incorporation into CL was reduced in F258V and PLSCR3 cells, yet ER MLCL AT activity was elevated, might suggest that mitochondrial MLCL AT may play the major role in the overall remodeling of mitochondrial CL. In contrast, ER MLCL AT may play a different role, possibly a functional role in CL remodeling as observed in apoptosis (Liu et al., 2003a,b).

In summary, alteration in expression of functional PLS3 alters the biosynthesis and remodeling of CL. Further studies are required to fully understand the effect of altered PLS3 levels on CL biosynthesis.

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