Published in final edited form as:
Apoptosis. 2008 July ; 13(7): 845–856. doi:10.1007/s10495-008-0219-4.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induced mitochondrial pathway to apoptosis and caspase activation is potentiated by phospholipid scramblase-3

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Abstract

Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) initiate pathways of cell death in which caspase activation is mediated either directly (without mitochondrial amplification), or indirectly via the release of apoptogenic factors from mitochondria. Phospholipid scramblases (PLS) are enzymes that play a key role in cellular function by inducing bidirectional movement of membrane lipids. Changes in mitochondrial membrane lipids, cardiolipin, are critical for mediating apoptotic response in many cell-types. PLS3 is a phospholipid scramblase that is localized to mitochondria and is thought to be involved in the regulation of apoptotic signals. Here we report that exogenous-expression of PLS3 enhances apoptotic death induced by TRAIL. This is acheived by potentiating the mitochondrial arm of the death pathway. Thereby, PLS3 expression facilitates changes in mitochondrial membrane lipids that promote the release of apoptogenic factors and consequent full activation and processing of the caspase-9 and effector caspase-3. Moreover, we show that knock-down of endogenous PLS3 suppresses TRAIL-induced changes in cardiolipin.

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Finally, we demonstrate that TRAIL-induced activation of PKC-delta mediates regulation of the PLS3-induced changes in cardiolipin.

Keywords

Apoptosis; TRAIL; Mitochondria; Phospholipases; Scramblase; Cardiolipin; PKC

Introduction

Apoptosis, a form of programmed cell death, is an active form of cellular destruction which, in concert with cell division, controls immune response, embryonic development and tissue homeostasis [1–6]. Apoptosis plays an important role in physiology; failure of this process can be harmful and disrupt the normal cellular state, thereby leading to many diseases including neoplastic transformation [1,7–9]. Apoptosis is mediated by caspases that are activated by either the death receptor (extrinsic) or mitochondrial (intrinsic) pathways. One death ligand is the Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL), a type II transmembrane protein and a member of the TNF family of ligands (Fas, TNF-alpha, etc.) [1,8]. TRAIL induces apoptosis in transformed or tumor, but not in normal, cells and hence has great promise as a tumor-selective anti-cancer agent [1,7,10–14]. Additionally, the functional expression of TRAIL has been shown on the surface of antigen presenting cells such as dendritic cells and monocytes that have been stimulated by cellular transformation, suggesting a role for TRAIL in the selective ablation of transformed cells before tumorigenesis [15–20]. The TRAIL receptors, DR4 and DR5, mediate TRAIL-induced apoptosis by recruiting, via death domain (DD) interaction, adaptor proteins such as Fas-Associated protein with Death Domain (FADD) and apical procaspases such as procaspase-8. This resulting assembly forms the Death Inducing Signaling Complex (DISC) [1,3,7,12,21,22], which promotes the auto-proteolytic activation and release of apical caspases [23,24]. In most tumor cells, which are considered to be type II cells, the activated apical caspases then initiate two main pathways of cell death [5,25–27]. In one pathway (the extrinsic), caspase-8 activation directly activates effector caspase-3, which facilitates apoptosis. Alternatively, the death receptor pathway can also activate the mitochondrial pathway (intrinsic pathway). In the mitochondrial pathway, changes in the mitochondrial membranes lead to the release of apoptogenic factors such as cytochrome *c* [5,7,22,28–30]. Upon entry into the cytosol, cytochrome *c* binds the caspase-activating protein Apaf1, stimulating the binding of Apaf1 to procaspase-9, forming apoptosome and subsequently inducing processing and activation of caspase-9. As a result, activated caspase-9 is then available to cooperate with caspase-8 in the processing and activation of caspase-3, leading to the demise of the cell.

There is emerging evidence that the lipid composition of mitochondrial membranes could modulate the membrane perturbing action of pro-apoptotic proteins like Bid [22,31–34]. Membrane lipid composition is altered by lipid-modifying, e.g. phospholipases, or lipid-transporting proteins [34]. Among the latter, phospholipid scramblases play an established role in the exposure of phosphatidylserine (PS) on the membrane surface of dying cells [32,33]. It is now recognized that there are four different members of the phospholipid scramblase family in humans and three in the mouse [26–30]. Phospholipid scramblase 1 has been reported to be involved in the externalization of phosphatidylserine from the cytosolic to the outer leaflet of the plasma membrane [31]. Conversely, phospholipid scramblase 3 (PLS3) is localized in mitochondria and may be involved in the regulation of mitochondrial function, especially by influencing the distribution of the mitochondria-specific lipid, cardiolipin [32,34,35].

To date, the involvement of PLS3 in regulating mitochondrial membrane lipids during TRAIL-induced cell death has not been elucidated. Death receptor induced signaling has been shown

to bring alterations in cardiolipin, a lipid unique to mitochondria [36]. Moreover, cardiolipin is instrumental for the action of death receptor-induced apoptotic signals at the mitochondrial membrane, since the pro-apoptotic action of tBid and Bax requires their interaction with this lipid and its metabolites [36–41]. The death-receptor induced changes in cardiolipin appear to follow an imbalance in the cellular homeostasis of phosphatidylcholin (PC) [36]. Metabolic alterations in PC also cause an elevation of diacylglycerol (DAG), which in turn activates PKCδ, an isoform of phospholipids-dependent ser/thr protein kinase [36]. Interestingly, scramblase-1 and -3 have been shown to be phosphorylated by PKCδ [34]. However, the involvement of PLS3 in regulating cardiolipin homeostasis and mitochondrial apoptotic pathway and consequently the role of PKCδ in modulating the function of PLS3 and its action on mitochondrial membrane during apoptosis is not known.

In this study, we show that PLS3 sensitizes Jurkat cells to TRAIL-induced apoptosis by promoting changes in the composition and distribution of cardiolipin. Hence, PLS3 enhances TRAIL-induced apoptosis by augmenting the release of apoptogenic factors and activating caspase-9, leading to the amplification and full activation and processing of caspase-3 and the downstream caspase cascade. Furthermore, we demonstrate an involvement of PKCδ in regulating this “apoptogenic” action of PLS3.

Materials and methods

Reagents

RPMI culture medium and fetal bovine serum (FBS) were purchased from Cellgro Mediatech Inc, (Herndon VA) and Biosource (Rockville, MD), respectively. Glutamine, penicillin, streptomycin and culture supplements were purchased from Gibco/BRL (Gaithersburg, MD). Caspase-8, caspase-9, caspase-3, Bid, HSP60, GRB2 and an antibody to PKCδ were obtained from BD Bioscience (Palo Alto, California). Anti-FLAG M2 agarose and anti-FLAG M2 were obtained from Sigma (St. Louis, MO). Anti-PLS3 antibody was purchased from Novus Biologicals, Inc(Littleton, CO). Recombinant human TRAIL was obtained from R&D Systems, Inc (Minneapolis, MN). The cardiolipin-sensitive probe 10-nonyl-acridine orange (NAO) was obtained from Molecular Probes (Eugene, OR).

Cell culture

Jurkat T cells (acute T cell leukemia) were purchased from the American Type Culture Collection (Rockville, MD), maintained in logarithmic growth, and were cultured in RPMI-1640. Cells were cultured in a density between 0.1 and 1.0×10^6/ml. Media was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in suspension at 37°C and 5% CO_2 in a humidified incubator and carried at $0.1–2.0 \times 10^6$ cells/ml, passaging two to three times weekly as needed. Cells were pelleted and resus-pended in fresh complete media in tissue culture plates 24 h before use in experiments to avoid any confounding gene expression that might occur because of handling.

Generation of PLS3 Jurkat-expressing cells

Full-length human PLS3 was kindly provided by Dr. Theresa Wiedmer, The Scripps Research Institute, La Jolla, CA. PLS3 was amplified using 30 cycles of denaturation (94°C, 1 min), annealing (50°C, 2 min), and extension (72°C, 3 min) using the following primers: Forward primer 5′TAGGATCCATGGCAGGCTACTTGCC3′; Reverse primer 5′ GACTCGAGCTAACTGGTGATGGCAG3′. The amplified PCR product was isolated, sequenced and sub-cloned into the pcDNA3.1FLAG vector (Invitrogen Life Technologies, Carlsbad, CA) between Bam H1 and Xho1 restriction sites. Western blotting was performed to confirm expression of PLS3 in stably transfected Jurkat cells (Fig. 1).

Generation of PLS3 mutant Jurkat-expressing cells

We generated mutant of PLS3 with a mutagenesis approach similar to that reported by Zhou et al. [47]. Zhou et al. found that mutation of Phe^{281} of the calcium-binding motif in PLS1 abolished function [47]. We altered the corresponding Phe^{258} in PLS3 to valine and generated stable transfectants of the mutant PLS3(F258V) in Jurkat cells.

Mitochondrial staining

The cardiolipin-sensitive probe 10-nonyl-acridine orange (NAO) was used to monitor changes in mitochondrial lipids in vivo. Briefly, after treatment with 10 ng/ml of recombinant human TRAIL/TNFSF10 (R&D systems, Minneapolis, MN), 10^6 cells were collected by centrifugation, washed and then resuspended in PBS containing 200 nM NAO (Molecular Probes, Eugene, OR) for 30 min at room temperature. Fluorescence was subsequently measured by flow cytometry using the Fl-1 channel. Using a similar protocol, we also measured mitochondrial membrane potential. 10^6 washed cells were resuspended in 1 ml PBS containing 25 nM CMXRos (Molecular Probes, Eugene, OR) or 40 nM TMRE (Molecular Probes, Eugene, OR) and after 30 min incubation at 37°C fluorescence was measured by flow cytometry with the FL-3 channel.

Tricyclodecan-9-yl xanthogenate (D609) was employed to pharmacologically inhibit phospholipase activity [36,42–44]. D609 was purchased from Biomol (Plymouth Meeting, PA). Cells were treated with D609 in buffered DMEM or RPMI medium for 30 min. prior to treatment with TRAIL.

PLS3 siRNA

Jurkat cells were transfected with PLS3 siRNA pool purchased from Dharmacon Inc (Chicago, IL) using electroporation. This pool contains four siRNAs against PLS3 (TAACATGTCTGCCAACAAT, GGAGAAGTCAGGCAT AGTA, GCAAATACAGGGAAGACTA, GGACGACTGCT CCAGCTTA, pool number = M-010225–00). The control siRNA was also purchased from Dharmacon (SIControl Non0Targetting SIRNA POOL #1 = D-001206-13-20). Briefly, 5×10^6 Jurkat cells were transfected with 100 nM/ml siRNA or negative control siRNA in serum-reduced medium. 24 h post-transfection, cells were replaced with 10% completed medium for overnight. Each 1×10^6 cells were then treated with TRAIL for 1 h in complete medium. Silencing of PLS3 was assessed by western blotting and NAO shift were measured by FACS.

Isolation of mitochondria

Mitochondria were isolated using a kit from Pierce Laboratories (Rockford, IL) with minor modifications. Briefly, Jurkat cells (2×10^7) were pelleted at ~850*g* for 2 min by centrifugation. The supernatant was removed and discarded. 800 μl of Mitochondria Isolation Reagent A was added and the tubes were vortexed at medium speed for 5 s and then incubated on ice for 2 min. 10 μl of Mitochondria Isolation Reagent B was added and the samples were vortexed at maximum speed for 5 s using an Eppendorf benchtop microcentrifuge. Mitochondria Isolation Reagent C was added and tubes were inverted several times to mix. The samples were centrifuged at 700*g* for 10 min at 4°C. The supernatant was transferred to a new, 2.0 ml tube and centrifuged at 12,000*g* for 15 min at 4°C. The supernatant, containing the cytosolic fraction, was transferred to a new tube and the remaining mitochondria pellet was washed with 500 μl of Mitochondria Isolation Reagent C. The mitochondria fraction was stored at −80°C until used.

Electron microscopy analysis

About 2×10^6 cells were fixed in 2% PFA/2.5% glutaraldehyde in 0.1 M Sodium cacodylate buffer, pH 7.4 for 1 h at room temperature, postfixed in 1% osmium tetroxide/1.5% potassium ferrocyanide in water for 30 min, stained in 1% uranyl acetate in maleate buffer pH 5.2 for 30 min at room temperature. After dehydration in graded ethanols (70%, 95% and 2 × 100%) cells were pelleted at 3,000 rpm for 3 min. Pellets were infiltrated in a 1:1 mixture of propylene oxide and Epon (TAAB Epon, Marivac Ltd, Nova Scotia, and Canada) for 2 h at room temperature, transferred to embedding molds filled with pure TAAB Epon and polymerized for 48 h at 60°C. Ultra thin sections (~80–90 nm) were mounted on copper grids, stained with 0.2% lead citrate and examined in a JEOL 1200EX transmission electron microscope.

Cell viability and Sub G1 apoptosis assay

Flow cytometry was used to assess Sub G1 DNA content in an entire cell population. 2×10^5 cells were seeded in 24-well plates and cultured for 24 h prior to treatment. For the inhibition assay, cells were pretreated with D609 inhibitors for 30 min. Cells were then treated with 10 ng/ml of TRAIL at varying time periods in 0.5% FBS medium. Cells were harvested, washed once with PBS and suspended in 400 μl propidium iodide (PI) solution (propidium iodide 50 μg/ml, 0.1% sodium citrate and 0.1% Triton-X 100). The Sub G1 DNA content was determined using a Becton Dickinson Flow Cytometer.

Western blot analysis

For Western analysis, 2×10^6–5×10^6 cells were cultured at a density of 1.0×10^6/ml and stimulated. Cells were harvested and pelleted in an Eppendorf microcentrifuge (1,200*g*, 5 min, 4°C), washed in 1×PBS and resuspended in a cell lysis buffer containing 20 mM Tris (pH 8.0), 0.5% (w/v) Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM dithiothreitol, 1 mM PMSF and 0.1 M NaCl. After 20 min. incubation at 4°C, supernatants were clarified (8,000*g*, 5 min, 4°C) and total protein concentration was determined by the method of Bradford and Lowry [7] using Bio-Rad Protein Assay reagents in a microtiter assay. 30 μg of total cellular protein was electrophoresed on a polyacrylamide SDS-PAGE gel and then transferred to a polyvinylidine difluoride membrane (Amersham, Arlington Heights, IL) by electroblotting overnight (25 mM Tris (pH 8.3), 192 mM glycine, 20% (v/v) methanol, 15 V, 100 mA, 4°C). Membranes were blocked with 10% (w/v) electrophoresis-grade biotin-depleted non-fat dry milk (Bio-Rad) in 1 × PBS, rinsed in PBS, probed with monoclonal mouse anti-human caspase-8, -9, -3, Bid, Cyt c or PKCδ(Pharmingen) at a 1:500 dilution and washed 3×in PBS. The secondary antibody was HRP-conjugated goat anti-mouse or anti-rabbit whole IgG used at 1:1,000 and 1:5,000, respectively (Transduction Laboratories, San Diego, CA) for 1 h at room temperature. The protein bands were then visualized using an enhanced chemiluminescence (ELC) detection system (Amersham Bioscience, Piscataway, NJ).

Statistics

All data were analyzed as continuous variables. Studies have shown the t-test to be robust enough to stand considerable departures from its theoretical assumptions, but with sample sizes $N \leq 5$ replications per treatment group in this study, the large sample assumption of the two-sample t-test was not satisfied [45]. The Wilcoxon rank sum test for two samples, a nonparametric analogue to the t-test test for two independent samples was applicable under the present setting and was used to compare treatment group medians [46]. A two-sided significance level of $P < 0.10$ was considered statistically significant instead of the commonly used significance level of $P < 0.05$. The less restrictive significance level was selected to counter-balance not only the small treatment group sample sizes but to take into consideration that the Wilcoxon test, as a nonparametric procedure has in general, greater probability relative to the t-test of failing to reject a false null hypothesis (greater probability of committing type

II error). Actual observed *P*-values of the Wilcoxon rank sum test were presented in figures. We decided a priori not to implement Boneferoni-type significance level adjustment to account for multiplicity. Firstly, we viewed the hypotheses we tested to be independent therefore not warranting adjustment of comparison-wise significance level. Secondly, there are different and opposing viewpoints over comparison-wise adjustments [47].

Results

Expression of PLS3 in Jurkat cells augments TRAIL-induced apoptosis

We previously demonstrated that TRAIL induces alterations in mitochondrial membrane lipid, cardiolipin (CL) [36]. As PLS3 is localized to the mitochondrion [34,36,40], we hypothesized that it may influence TRAIL-induced apoptosis by regulating the mitochondrial lipid membrane. Similar to previous reports [19,48], we observed that TRAIL-induces apoptosis in a time- and dose-dependent manner (Fig. 1a), and that a concentration of TRAIL at 10 ng/ml applied over a time period of 6 h causes complete proteolytic cleavage of caspase-8 in Jurkat cells (Fig. 1a). Therefore, throughout the remainder of this paper, TRAIL treatment should be assumed to involve the aforementioned concentration and time period unless if otherwise noted.

In order to investigate the role of PLS3 in TRAIL-induced apoptosis, we generated a Jurkat T lymphocytic cell line with ectopic expression of PLS3 (F-PLS3) (Fig. 1b). We then ascertained that F-PLS3 is localized to the mitochondrion by generating cytosolic and mitochondrial fractions and examining the intracellular distribution pattern of PLS3 by Western blotting with an anti-FLAG antibody. These studies demonstrated that F-PLS3, in accordance with other reports for endogenous PLS3 [35,49], was localized exclusively to the mitochondrial, and not the cytosolic fraction (Fig. 1c). The mitochondrial and cytosolic markers, Hsp60 and GRB2 respectively, were used as controls to demonstrate the purity of the fractions [36].

We then sought to determine whether F-PLS3 expression enhances TRAIL-induced apoptosis. The Sub G1 DNA content assay demonstrated that, although there was less than 2% death in untreated parental Jurkat cells or Jurkat cells with ectopic expression of F-PLS3, treatment of parental cells with TRAIL resulted in 15% cell death (Fig. 1d). Interestingly, treating cells expressing F-PLS3 with TRAIL resulted in 32% cell death (Fig. 1d), indicating that F-PLS3 sensitizes Jurkat cells to TRAIL-induced apoptosis. On the other hand, Jurkat cells transfected with a non functional mutant of F-PLS3 (F258V) did not sensitize Jurkat cells to TRAIL-induced apoptosis (Fig. 1e).

PLS3 expression in Jurkat cells compromises mitochondrial integrity following TRAIL treatment

Cardiolipin (CL) present in mitochondrial membranes is highly susceptible to peroxidation and/or degradation in response to apoptosis [37,50] and modulates the action of Bid, a BH3-only member of the Bcl-2 family which cooperates with either Bak or Bax in forming proteolipid pores in the outer mitochondrial membrane [34,35,41]. However, the signaling events responsible for the changes in cardiolipin that prime mitochondria for the pro-apoptotic action of death ligands are not well characterized. In order to determine whether F-PLS3 activity also enhanced TRAIL-induced modification of cardiolipin [33], we first monitored the cardiolipin status within live cells using conventional staining with the specific dye, NAO [33].

NAO staining (Fig. 2a, b) revealed that treatment of parental Jurkat cells with TRAIL for 1 h resulted in a 15% decrease in NAO staining. Interestingly, TRAIL resulted in a greater than 30% decrease in NAO staining in F-PLS3 expressing cells (Fig. 2a). We have recently reported

that an imbalance in the cellular homeostasis of phosphatidylcholine (PC) caused by altered phospholipase activity results in TRAIL-induced alterations in cardiolipin [5, 36] Therefore, in order to determine whether phospholipase activity is involved in the F-PLS3 mediated changes in cardiolipin, we utilized a pharmacological inhibitor of phospholipases known as D609 [42]. This compound has been generally considered to inhibit PC-PLC as well as PLD activity and has been shown to protect cells from Fas-induced apoptosis as well as TRAIL-induced apoptosis [36, 42–44]. As shown in Fig. 2a, b, the F-PLS3-induced changes in NAO staining were attenuated by D609 indicating that PLS3 can mediate early changes in mitochondrial cardiolipin content that is dependent on phospholipase C and D activity. We further investigated cardiolipin changes in response to TRAIL treatment using a siRNA pool against PLS3 to silence PLS3 (Fig. 2c upper panel). NAO staining revealed that TRAIL-induced cardiolipin changes during apoptosis was suppressed in Jurkat cells transfected with siRNA against PLS3 compared with Jurkat cells transfected with scrambled siRNA control (Fig. 2c lower panel) from 15.25 to 8.16 which is 46.5% reduction. These observations indicate that TRAIL-induced changes in cardiolipin (change the mitochondrial potential) is mediated at least partially a PLS3-dependent manner. Moreover, our data suggests that cardiolipin externalization precedes activation of execution caspases, as 1 h of TRAIL treatment did not result in high levels of apoptosis (Fig. 2d), while there was significant level of cardiolipin remodeling (as measured by NAO staining) at this time point (Fig. 2b).

We next investigated the morphology of mitochondria in these cells by electron microscopic analysis. As shown in Fig. 2e, F-PLS3 expression causes some changes in the morphology of mitochondria. Notably, when F-PLS3 expressing cells were treated with TRAIL (1 h), there was a significant morphological change in the mitochondria as compared with parental cells. As shown in Fig. 2e, F-PLS3 expressing cells that were treated with TRAIL exhibited an apparent disruption of the inner membrane and cristae. Micrographs are shown at the same magnification. Together, these studies demonstrate that PLS3 expression alters the integrity of mitochondrial membrane that is dependent on activity of phospholipases.

PLS3 expression in Jurkat cells enhances TRAIL-mediated activation of the mitochondrial apoptotic pathway

Localization of PLS3 to the mitochondrion and its involvement in translocation of cardiolipin indicates that PLS3 may be a critical component for the initiation and/or reinforcement of cell death pathways at mitochondria. Thus, we sought to determine whether expression of PLS3 potentiated the activation of apoptotic events downstream of mitochondria, such as the release of apoptogenic factors (i.e., cyt *c*) and the activation of caspase-9. As shown in Fig. 3, we found that while there was no release of cytochrome *c* from the mitochondria to the cytosol in parental and F-PLS3-expressing cells, TRAIL treatment of parental cells for 6 h resulted in minimal release of cytochrome *c*, but significant increase in cytochrome *c* release was detected by Western blot analysis in F-PLS3-expressing cells that were treated with TRAIL (Fig. 3). These data establish the enhancement of apoptotic effect of TRAIL by PLS3 in Jurkat cells at the level of the mitochondria.

Activation of the mitochondrial apoptotic pathway and release of cyt c leads to processing of caspase-9 within the apoptosome [6,25]. While TRAIL treatment for 6 h resulted in incomplete processing of procaspase-9 in parental cells, it promoted enhanced cleavage of procaspase-9 in cells expressing F-PLS3 (Fig. 3b). This was further supported by a caspase-9 activity assay, where TRAIL treatment resulted in 9-fold activation of caspase-9 in F-PLS3-expressing cells, compared with 3-fold activation in parental cells (Fig. 3c). Moreover, the caspase-9-inhibitor, LEHD blocked the F-PLS3 mediated activation of caspase-9 (Fig. 3c). These studies demonstrate that PLS3 does indeed cooperate with TRAIL in activation of caspase-9.

We next evaluated whether expression of F-PLS3 changes the kinetics of caspase-9 processing. For this, a time course analysis of the activation of caspase-9 was carried out in parental and PLS3-expressing cells. Using Western blotting (Fig. 3d) we demonstrated that there was a shift in the time course of caspase-9 activation in cells expressing PLS3, compared to parental cells. Caspase-9 activation and cleavage in F-PLS3-expressing cells was observed in as early as 3 h, compared to the 6 h necessary for cleavage of caspase-9 in parental cells.

These studies demonstrate that F-PLS3 promotes early mitochondrial changes in response to death receptor-induced apoptosis, hence facilitating both the release of apoptogenic factors and the full activation and processing of caspase-9.

PLS3 expression in Jurkat cells enhances TRAIL-mediated activation of caspases-3 and -8

It has been previously demonstrated that caspase-9 activation leads to the processing and activation of caspase-3 [25,36,40,51]. Additionally, caspase-3 appears to be an essential component of the apoptotic machinery in many cell types and causes activation of a cascade of executioner caspases leading to the demise of the cell [13,29,52]. As PLS3 promotes the activation of caspase-9, we next evaluated the caspase-3 processing in F-PLS3-expressing cells. Here we demonstrated by western blot analysis (Fig. 4a) that TRAIL treatment resulted in complete activation and cleavage of procaspase-3 only in cells expressing F-PLS3. This suggests that PLS3 enhances TRAIL-induced activation of caspase-3 in TRAIL-induced apoptosis. This was further supported by a caspase-3 activity assay, where TRAIL treatment resulted in 28-fold activation of caspase-3 in F-PLS3-expressing cells, compared with 9-fold activation in parental cells (Fig. 4b).

In addition to the activation of executioner caspases, caspase-3 has also been shown to reinforce activation of the initiator caspase-8 via a feedback amplification loop [52]. Since caspase-8 has been implicated in TRAIL-induced apoptosis by TRAIL receptors (DR4 and DR5) [53], we sought to determine whether expression of F-PLS3 enhanced the TRAIL-induced extrinsic pathway by affecting caspase-8 activation and processing. Our data demonstrate that TRAIL treatment of parental cells resulted in incomplete processing of procaspase-8, whereas treatment of cells expressing F-PLS3 with TRAIL resulted in significant processing of caspase-8, generating the signature fragment p20 band (Fig. 5a). This was further supported by a caspase-8 activity assay, where TRAIL treatment of parental cells alone resulted in 3-fold activation of caspase-8, while expression of F-PLS3 in these cells led to a 14-fold activation of caspase-8 upon TRAIL treatment (Fig. 5b). Furthermore, a time course analysis revealed that significant activation and processing of cas-pase-8 took place within 3 h in TRAIL-treated cells expressing F-PLS3, whereas in parental cells, caspase-8 activation and processing was observed at 6 h following TRAIL treatment (Fig. 5c).

Finally, as activation of caspase-8 results in the processing of Bid, which is a preferred substrate of caspase-8, we also evaluated the cleavage of Bid [50]. Our results indicate that while there was incomplete cleavage of Bid in TRAIL-treated parental cells, treatment of cells expressing F-PLS3 resulted in complete Bid cleavage by 6 h (Fig. 5d). This suggests that PLS3 also enhances TRAIL-induced Bid cleavage in Jurkat cells.

PLS3 enhances TRAIL-dependent PKCδ cleavage and cell death

PKCδ is a member of the PKC superfamily of proteins that is activated by diacylglycerol (DAG) [54]. Some isoforms of the PKC family are activated in response to various stimuli, including apoptosis [55]. Activation of PKCδ by apoptotic stimuli results in the translocation of PKCδ to the mitochondrion [55,56]. We previously reported that TRAIL signaling induces an alteration in mitochondrial membrane lipids, in particular cardiolipin and have unveiled a link between TRAIL signaling and alteration of membrane lipid homeostasis [36]. Specifically,

we have demonstrated that TRAIL-induced changes in cardiolipin followed a disparity in the cellular homeostasis of phosphatidylcholine (PC), leading to an elevation in diacylglycerol (DAG). Increased DAG in turn phosphorylates and activates PKCδ([36]. Moreover, PLS3 amino acid sequence contains a conserved PKCδ(phosphorylation site [57], and PLS3 has been shown to be a high affinity substrate of PKCδ([34,58]. As, PKCδ activation is dependent on caspase mediated cleavage resulting in the release of a constitutively active kinase domain [45], we hypothesized that TRAIL-induced regulation of PKCδ might be involved in enhancement of apoptosis by PLS3. In order to test this premise, we analyzed the TRAIL-induced cleavage of PKCδ in the parental as well as F-PLS3-expressing Jurkat cells. Figure 6 demonstrates an enhancement of PKCδ(cleavage in F-PLS3-expressing cells (Fig. 6a) and this is accompanied by an increase in the apoptotic response of these cells to TRAIL (40% as compared to 20% cell death in parental Jurkat cells) (Fig. 6b). Thus, the TRAIL-induced activation of PKCδ regulates the function of PLS3 leading to the modulation of the mitochondrial lipid membrane and augmentation of apoptotic machinery.

Discussion

TRAIL is a promising tumor selective cancer therapeutic [10–14,20]. However, certain tumor cells are resistant to TRAIL [10–14,20]. Significantly, mitochondria constitute an important target in overcoming inherent resistance to TRAIL in many types of tumors (i.e., colon carcinomas and gliomas) [5,21,28]. These observations suggest that a block in the activity of TRAIL-induced mitochondrial pathways may prompt some tumor cells to become TRAIL-resistant. A better understanding of the mechanisms by which mitochondrial apoptosis machinery is regulated could provide insights into how resistance could be instigated. Moreover, these studies will provide targets for therapeutic agents that alone or in combination with current therapy could overcome the therapeutic resistance. Thus, herein, we have investigated the role of PLS3 in regulating the mitochondrial death machinery in type II cells, which require mitochondria for mediating cell death [21,22].

Our studies demonstrate that PLS3 accelerates the alteration in cardiolipin homeostasis, perhaps by facilitating its translocation from the inner membrane to the outer face of mitochondria, where it can be re-modeled and modified also by non-mitochondrial enzymes [32]. This surface exposure of cardiolipin (and its metabolites) onto the outer mitochondrial membrane is thought to be essential for its interaction with tBid and Bax and their cooperation to form supramolecular openings in the outer mitochondrial membrane [32–37]. Consequently, our study provides evidence that PLS3 is critical for maintaining a proper flow of mitochondrial lipids to fuel the normal signaling of cell death at the mitochondrial level. The augmentation of apoptotic capacity of TRAIL in cells over-expressing PLS3 resulted in increased release of apoptogenic factors and thus amplification of the activity of caspases. Additionally, we demonstrated that TRAIL-induced processing and activation of PKCδ facilitates cardiolipin degradation by enhancing its scramblase-mediated shuttling to the mitochondrial surface.

In conclusion, these results indicate that TRAIL stimulation induces changes in PKCδ, regulates PLS3 activity and affect mitochondrial membranes and can contribute to apoptosis via both caspase-9 signaling as well as activation of caspase-8 via signaling through an amplification loop (Fig. 7). The TRAIL-induced signaling pathways that regulate PKCδ, PLS3 and cardiolipin will thus offer novel targets for anti-cancer drugs that, by enhancing mitochondrial pathway to apoptosis could promote apoptosis of cancer cells that have become resistance to chemotherapy.

Acknowledgments

We thank Drs. Wiedmer and Ohno for scramblase and PKCδ constructs, respectively. This work was supported by NIH grants CA105306 and HL080192 to RKF. RKF is an American Cancer Society Scholar. KN was supported by NIH 5 T32 HL07893 and UNCF/MERCK.

References

1. Abe K, Kurakin A, Mohseni-Maybodi M, Kay B, Khosravi-Far R. The complexity of TNF-related apoptosis-inducing ligand. Ann NY Acad Sci 2000;926:52–63. [PubMed: 11193041]
2. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 2003;10:66–75. [PubMed: 12655296]
3. MacFarlane M. TRAIL-induced signalling and apoptosis. Toxicol Lett 2003;139:89–97. [PubMed: 12628743]
4. Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. Int J Biochem Cell Biol 2004;36:2405–2419. [PubMed: 15325581]
5. Khosravi-Far R, Esposti MD. Death receptor signals to mitochondria. Cancer Biol Ther 2004;3:1051–1057. [PubMed: 15640619]
6. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001;15:2922–2933. [PubMed: 11711427]
7. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003;10:26–35. [PubMed: 12655293]
8. Golstein P. Cell death: TRAIL and its receptors. Curr Biol 1997;7:R750–R753. [PubMed: 9382834]
9. Lockshin RA, Zakeri Z. Cell death in health and disease. J Cell Mol Med 2007;11:1214–1224. [PubMed: 18031301]
10. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–162. [PubMed: 10411544]
11. French LE, Tschopp J. The TRAIL to selective tumor death. Nat Med 1999;5:146–147. [PubMed: 9930856]
12. Ozoren N, El-Deiry WS. Cell surface Death Receptor signaling in normal and cancer cells. Semin Cancer Biol 2003;13:135–147. [PubMed: 12654257]
13. Thorburn A. Death receptor-induced cell killing. Cell Signal 2004;16:139–144. [PubMed: 14636884]
14. Song K, Benhaga N, Anderson RL, Khosravi-Far R. Transduction of tumor necrosis factor-related apoptosis-inducing ligand into hematopoietic cells leads to inhibition of syngeneic tumor growth in vivo. Cancer Res 2006;66:6304–6311. [PubMed: 16778207]
15. Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K, Yagita H. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J Exp Med 1999;189:1451–1460. [PubMed: 10224285]
16. Mackay F, Kalled SL. TNF ligands and receptors in autoimmunity: an update. Curr Opin Immunol 2002;14:783–790. [PubMed: 12413530]
17. Mariani SM, Krammer PH. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. Eur J Immunol 1998;28:1492–1498. [PubMed: 9603453]
18. Martinez-Lorenzo MJ, Alava MA, Gamen S, et al. Involvement of APO2 ligand/TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. Eur J Immunol 1998;28:2714–2725. [PubMed: 9754559]
19. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003;22:8628–8633. [PubMed: 14634624]
20. Zamai L, Ahmad M, Bennett IM, Azzoni L, Alnemri ES, Perussia B. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med 1998;188:2375–2380. [PubMed: 9858524]
21. Ozoren N, El-Deiry WS. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. Neoplasia 2002;4:551–557. [PubMed: 12407450]

22. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. Embo J 1998;17:1675–1687. [PubMed: 9501089]
23. Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. An induced proximity model for caspase-8 activation. J Biol Chem 1998;273:2926–2930. [PubMed: 9446604]
24. Yang X, Chang HY, Baltimore D. Autoproteolytic activation of pro-caspase by oligomerization. Mol Cell 1998;2:319. [PubMed: 9659928]
25. Boatright KM, Renatus M, Scott FL, et al. A unified model for apical caspase activation. Mol Cell 2003;11:529–541. [PubMed: 12620239]
26. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–656. [PubMed: 12209154]
27. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 1999;17:331–367. [PubMed: 10358762]
28. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–1308. [PubMed: 9721089]
29. Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326(Pt 1):1–16. [PubMed: 9337844]
30. Matarrese P, Gambardella L, Cassone A, Vella S, Cauda R, Malorni W. Mitochondrial membrane hyperpolarization hijacks activated T lymphocytes toward the apoptotic-prone phenotype: homeostatic mechanisms of HIV protease inhibitors. J Immunol 2003;170:6006–6015. [PubMed: 12794128]
31. Ouasti S, Matarrese P, Paddon R, et al. Death receptor ligation triggers membrane scrambling between golgi and mitochondria. Cell Death Differ 2007;14:453–461. [PubMed: 17008914]
32. Sahu SK, Gummadi SN, Manoj N, Aradhyam GK. Phospholipid scramblases: an overview. Arch Biochem Biophys 2007;462:103–114. [PubMed: 17481571]
33. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 2000;405:85–90. [PubMed: 10811223]
34. Liu J, Chen J, Dai Q, Lee RM. Phospholipid scramblase 3 is the mitochondrial target of protein kinase C delta-induced apoptosis. Cancer Res 2003;63:1153–1156. [PubMed: 12649167]
35. Liu J, Dai Q, Chen J, et al. Phospholipid scramblase 3 controls mitochondrial structure, function, and apoptotic response. Mol Cancer Res 2003;1:892–902. [PubMed: 14573790]
36. Sandra F, Degli Esposti M, Ndebele K, et al. Tumor necrosis factor-related apoptosis-inducing ligand alters mitochondrial membrane lipids. Cancer Res 2005;65:8286–8297. [PubMed: 16166305]
37. Esposti MD. The roles of Bid. Apoptosis 2002;7:433–440. [PubMed: 12207176]
38. Goonesinghe A, Mundy ES, Smith M, Khosravi-Far R, Martinou JC, Esposti MD. Pro-apoptotic Bid induces membrane perturbation by inserting selected lysolipids into the bilayer. Biochem J 2005;387:109–118. [PubMed: 15500442]
39. Kuwana T, Mackey MR, Perkins G, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 2002;111:331–342. [PubMed: 12419244]
40. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell 2003;112:481–490. [PubMed: 12600312]
41. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–730. [PubMed: 11326099]
42. Cifone MG, Roncaioli P, De Maria R, et al. Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J 1995;14:5859–5868. [PubMed: 8846779]
43. Exton JH. Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. Eur J Biochem 1997;243:10–20. [PubMed: 9030716]
44. Han JS, Hyun BC, Kim JH, Shin I. Fas-mediated activation of phospholipase D is coupled to the stimulation of phosphatidylcholine-specific phospholipase C in A20 cells. Arch Biochem Biophys 1999;367:233–239. [PubMed: 10395739]

45. Boneau C. The effects of violations of assumptions underlying the test. Psychol Bull 1960;57:49–64. [PubMed: 13802482]
46. Haldane J. The Wilcoxon and related tests of significance. Experientia 1956:205. [PubMed: 13330789]
47. Westfall PH, Krishen A, Young SS. Using prior information to allocate significance levels for multiple endpoints. Statistics Med 1998;17:2107–2119.
48. Kimberley FC, Screaton GR. Following a TRAIL: update on a ligand and its five receptors. Cell Res 2004;14:359–372. [PubMed: 15538968]
49. Liu J, Epand RF, Durrant D, et al. Role of phospholipid Scramblase 3 in the regulation of tumor necrosis factor-alpha-induced apoptosis. Biochemistry 2008;47:4518–4529. [PubMed: 18358005]
50. Esposti MD, Cristea IM, Gaskell SJ, Nakao Y, Dive C. Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. Cell Death Differ 2003;10:1300–1309. [PubMed: 12894218]
51. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–446. [PubMed: 9390553]
52. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999;6:1067–1074. [PubMed: 10578175]
53. Jin TG, Kurakin A, Benhaga N, et al. Fas-associated protein with death domain (FADD)-independent recruitment of c-FLIPL to death receptor 5. J Biol Chem 2004;279:55594–55601. [PubMed: 15485835]
54. Kikkawa U, Matsuzaki H, Yamamoto T. Protein kinase C delta (PKC delta): activation mechanisms and functions. J Biochem (Tokyo) 2002;132:831–839. [PubMed: 12473183]
55. Brodie C, Blumberg PM. Regulation of cell apoptosis by protein kinase C delta. Apoptosis 2003;8:19–27. [PubMed: 12510148]
56. Sumitomo M, Ohba M, Asakuma J, et al. Protein kinase c delta amplifies ceramide formation via mitochondrial signaling in prostate cancer cells. J Clin Invest 2002;109:827–836. [PubMed: 11901191]
57. Zhao J, Zhou Q, Stout JG, Luhm RA, Wiedmer T, Sims PJ. Molecular cloning of human plasma membrane phospholipid scrambalase. J Biol Chem 1997;272:18240. [PubMed: 9218461]
58. He Y, Liu J, Grossman D, et al. Phosphorylation of mitochondrial phospholipid scramblase 3 by protein kinase C-delta induces its activation and facilitates mitochondrial targeting of tBid. J Cell Biochem 2007;101:1210–1221. [PubMed: 17226776]

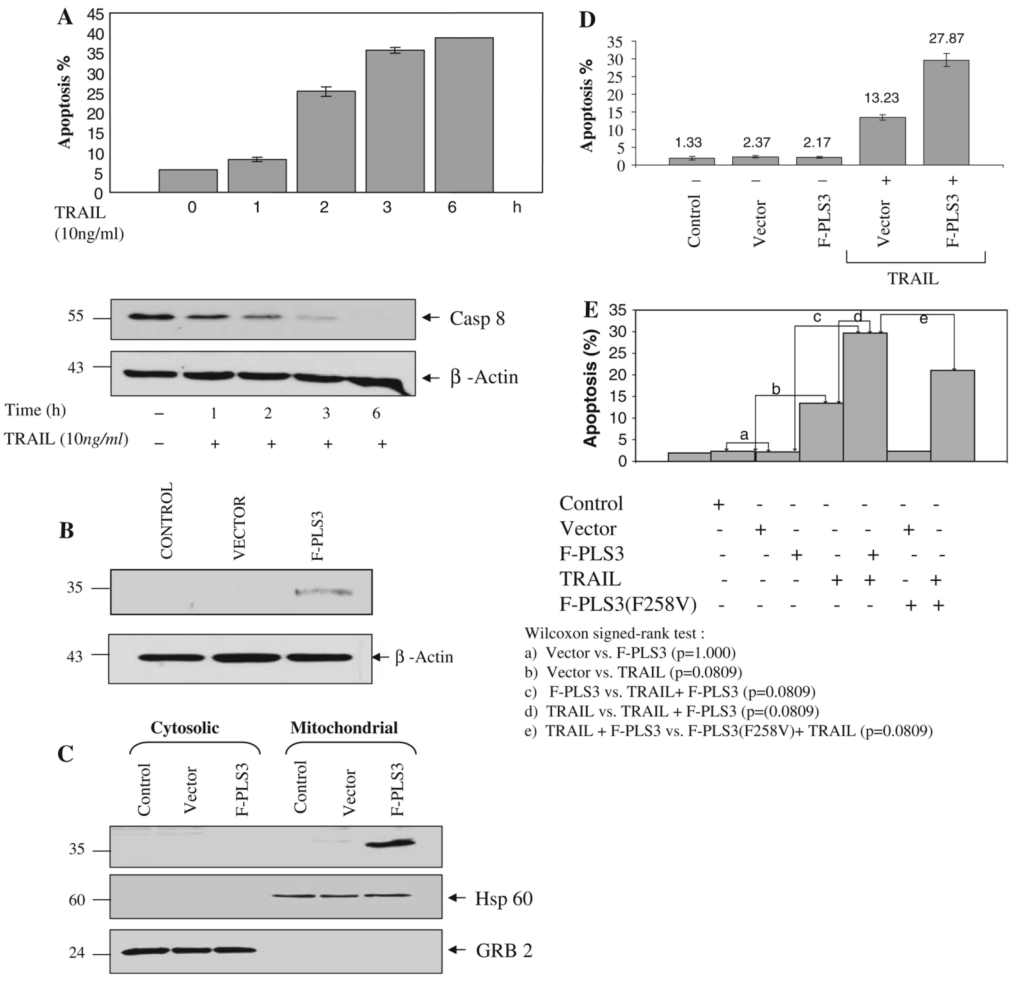

Fig. 1.

PLS3 is localized to mitochondria and enhances TRAIL-induced apoptosis. (**a**). TRAIL activates caspase-8 and induces apoptosis in a time-dependent manner. Jurkat cells were treated with 10 ng/ml TRAIL for 0, 1, 2, 3 and 6 h. The apoptotic, subG1, population were measured by FACS analysis. Western blot analysis was carried out to measure caspase-8 activation. (**b**) PLS3 is ectopically expressed in Jurkat cells. Western blot analysis confirming expression of PLS3 in stably transfected Jurkat cells. *β*-actin was used as a loading control. (**c**) PLS3 is predominantly localized to the mitochondria. Western blotting indicates that expression of PLS3 is limited to the mitochondrial rather than the cytosolic fraction isolated from Jurkat cells. Hybridizations with antibodies to Hsp60 and GRB2 were used as loading controls for the mitochondrial and cytosolic fractions, respectively. (**d**) PLS3 sensitizes Jurkat cells to TRAIL-induced apoptosis. Jurkat cells stably transfected with pc DNA 3.1 FLAG or pcDNA 3.1 FLAG PLS3 were treated with TRAIL for 6 h. Jurkat cells were then recovered and stained with propidium iodide (Roche, USA). Sub G1 DNA content was analyzed by FACS using the fluorescent channel 1 (FL-1). Expression of PLS3 significantly enhanced apoptosis by more than two folds. (**e**) Expression of mutant PLS3(F258V) reduced TRAIL-mediated apoptosis to 20% as compared to 32% in PLS3 expressing Jurkat cells

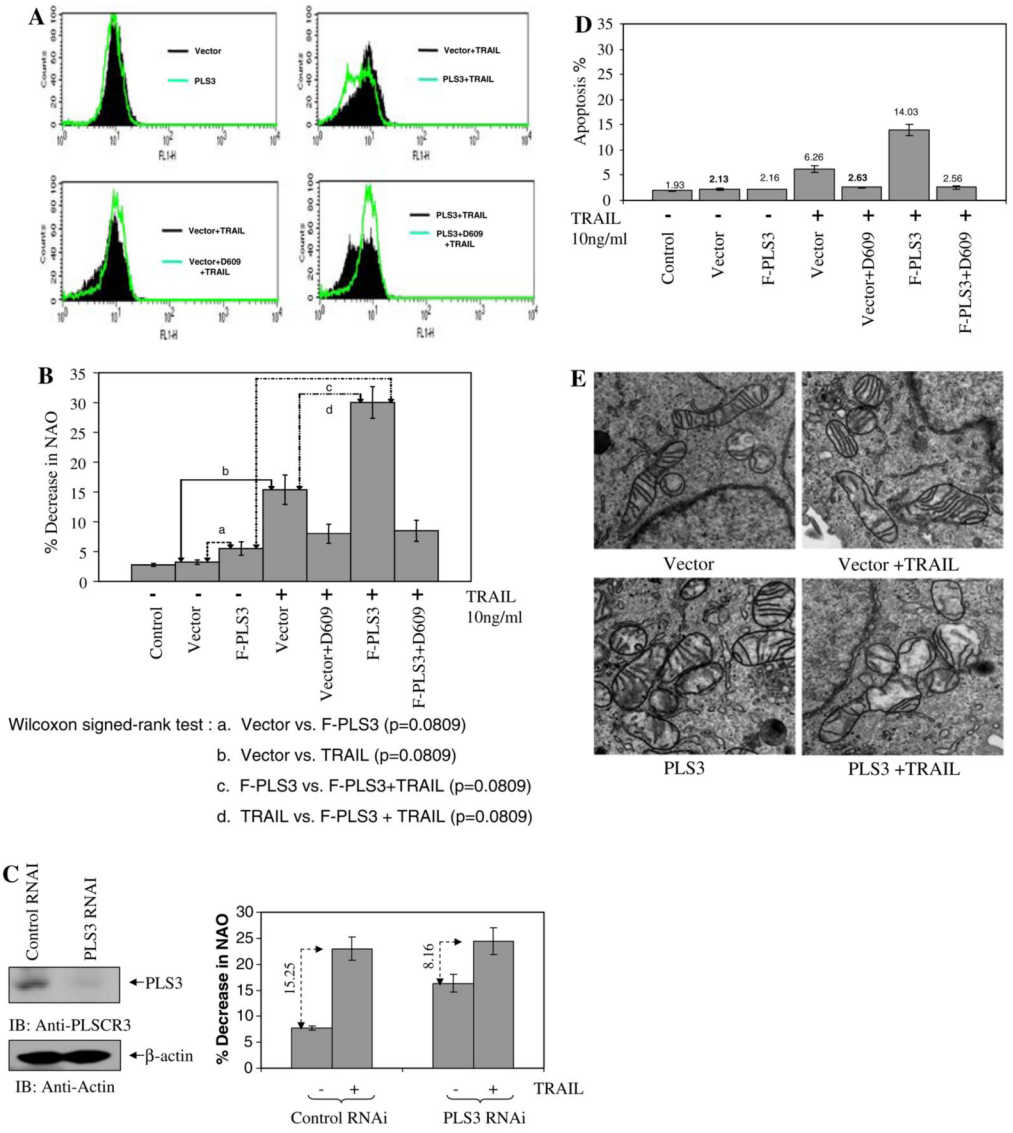

Fig. 2.

PLS3 expression promotes scrambling of mitochondrial membrane lipid. Jurkat cells, either parental or stably transfected with PLS3, were treated with TRAIL for 1 h. Cells were then recovered and stained with 10-N-Nonyl acridine orange (NAO), a highly specific stain for cardiolipin in the mitochondria. (**a**–**b**) NAO assay analysis of cardiolipin. Expression of PLS3 significantly alters TRAIL-induced NAO staining to 30% inhibition as compared with 15% inhibition in parental cells treated with TRAIL. A pharmacological inhibitor or phospholipases, D609, blocked this change of NAO-staining. (**c**) Western blotting analysis showing knock-down of PLS3 siRNA-treated Jurkats cells. Actin is included as a loading control (Fig. 2c upper panel). Cardiolipin changes during apoptosis was suppressed in Jurkat cells transfected with siRNA against PLS3 compared with Jurkat cells transfected with scrambled siRNA control (Fig. 2c lower panel) from 15.25 to 8.16 which is 46.5% reduction. (**d**) Apoptosis was assessed by DNA content analysis by means of flow cytometry. PLS3 significantly enhanced TRAIL-mediated apoptosis to 15% as compared to 5% in parental cells and this enhancement was blocked by D609. (**e**) Electron microscopic analysis of mitochondria structure. Parental cells and PLS3 expressing cells treated with or without 10 ng/ml TRAIL for 2 h. Irregular

mitochondrial appearance and loss of cristae was detected in PLS3 expressing cells. The micrographs are shown at the same magnification

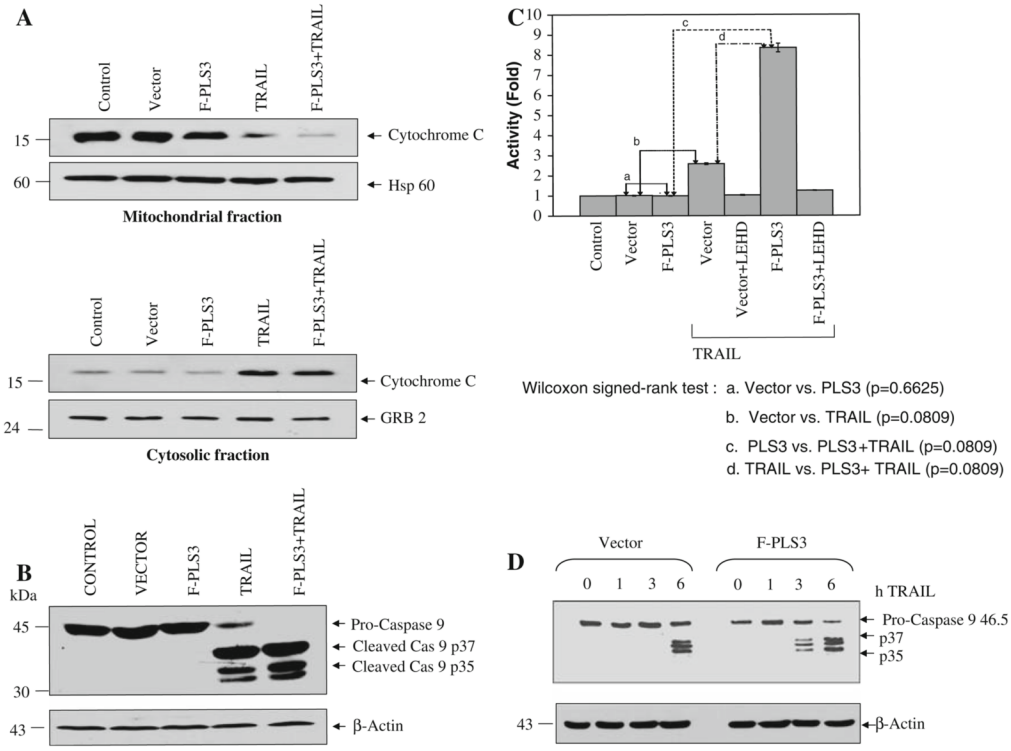

Fig. 3.

PLS3 expression enhances TRAIL-mediated activation of the mitochondrial apoptotic pathway. (**a**) Expression of PLS3 enhanced the TRAIL-mediated release of cytochrome *c* from the mitochondrion to the cytoplasm. Mitochondrial and cytoplasmic fractions of TRAIL-treated PLS3 stably transfected Jurkat cells were analyzed by Western blotting with antibodies against cytochrome *c* (mouse anti cytochrome *c*, clone 7H8.2C12. BD Bioscience). Staining for Hsp 60 and GRB 2 were used as loading controls for the mitochondrial and cytoplasmic fractions, respectively. Data shown are representative of 3 independent experiments. (**b**) PLS3 enhances the TRAIL-induced proteolytic cleavage of caspase-9. PLCRS3 stably transfected Jurkat cells were treated with TRAIL for 6 h. Western blot analysis was performed with antibodies to procaspase-9. (Human Specific. Cell Signaling cat # 9502). The molecular mass of the inactive pro-form of the caspase-9 (48 kD) is indicated by arrows. (**c**) Caspase-9 activity was analyzed using a caspase-9 fluorescent assay to confirm the Western blotting results. TRAIL treatment of parental cells resulted in a 3-fold activation of caspase-9, and yielded a 9-fold activation of caspase-9 when PLS3 was expressed. A caspase-9 inhibitor (LEHD) was used to demonstrate that the activity of PLS3 was caspase-9-specific. (**d**) Time course analysis (0, 1, 3 and 6 h) of activation of caspase-9 in TRAIL-treated PLCRS3 stably transfected Jurkat cells. Expression of PLS3 in Jurkat cells resulted in a significant change in the time course of caspase-9 processing

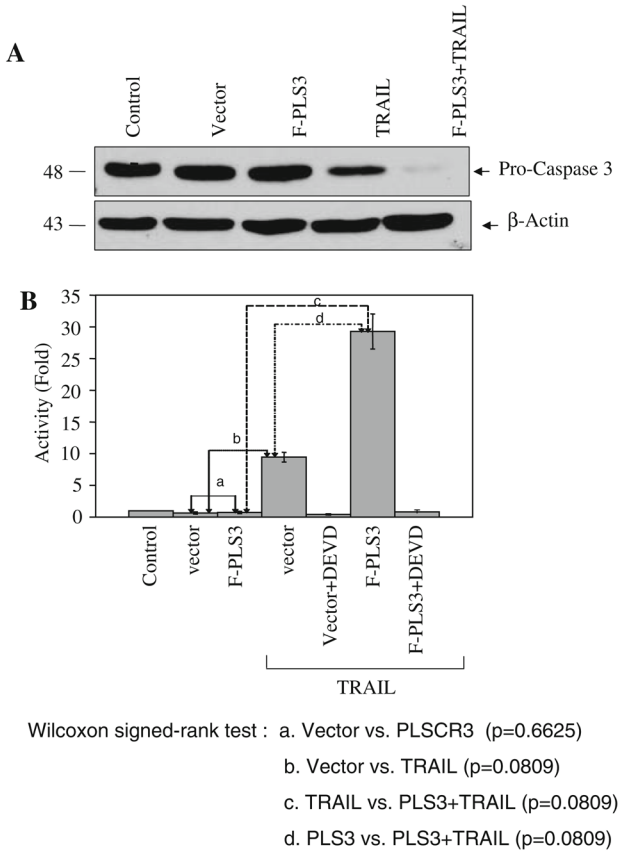

Fig. 4.

PLS3 enhances TRAIL-mediated activation of caspases 3. (**a**) PLS3 enhances TRAIL-induced activation and proteolytic cleavage of the effector caspase-3. PLCRS3 stably transfected Jurkat cells were treated with TRAIL for 6 h. Western blot analysis with antibodies to procaspase-3 (rabbit anti caspase 3 polyclonal. BD Bioscience cat # 552785) demonstrated that PLS3 significantly enhanced TRAIL-induced proteolytic cleavage of caspase-3. The molecular mass of the inactive proform of procaspase-3 (32 kDa) is indicated (arrows). *β*-actin was used as a loading control. (**b**) Caspase-3 activity was analyzed in Jurkat cells using a caspase fluorescent assay kit to confirm the Western blot results. TRAIL treatment resulted in a 10-fold activation of caspase-3 in parental cells, and a 30-fold activation in PLS3-expressing cells. A caspase-3 inhibitor (DEVD) was used to demonstrate that the activity of PLS3 was caspase-3-dependent

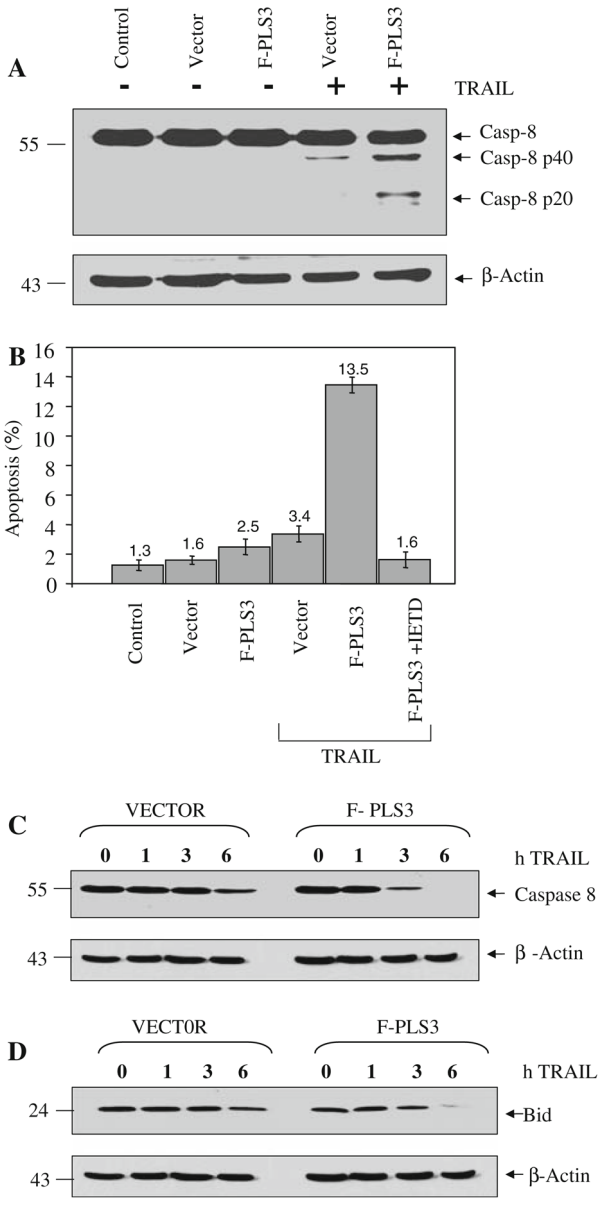

Fig. 5.

Expression of PLS3 enhances the activation of caspase-8 in TRAIL-treated cells. (**a**) Western blot analysis of Jurkat cells demonstrated that PLS3 enhanced TRAIL-induced proteolytic cleavage of caspase 8. The molecular mass of the inactive proform (55 kDa) of caspase 8 is indicated (arrow). (**b**) A caspase-8 fluorescent activity assay confirmed the Western blot results and demonstrated that expression of PLS3 significantly enhanced the TRAIL-induced activation of caspase-8. TRAIL treatment of parental cells resulted in a 3-fold activation of caspase-8, while expression of PLS3 in these cells yielded a 14-fold activation of caspase-8 upon TRAIL treatment. A caspase-8 inhibitor (IETD) was used to show that the activity of PLS3 was caspase-8-dependent. (**c**) Ectopic expression of PLS3 in Jurkat cells enhances TRAIL-induced activation and proteolytic cleavage of the initiator caspase-8. Jurkat cells stably transfected with pcDNA 3.1 FLAG or pcDNA 3.1 FLAG PLS3 were treated with TRAIL for 6 h. Western blot analysis with antibodies to procaspase-8 demonstrated that complete cleavage of caspase-8 is observed by the 6th hour only in cells with ectopic expression of PLS3.

The molecular mass of the inactive proform (57 kDa) of the procaspase-8 is indicated (arrows). *β*-actin was used as a loading control. (**d**) TRAIL-induced Bid cleavage is augmented in PLS3 expressing cells. Western blot analysis of Bid cleavage (rabbit anti BID polyclonal, BD Bioscience cat # 350365) over the course of 6 h in PLS3 stably transfected Jurkat cells treated with TRAIL indicates that PLS3 expression in Jurkat cells enhanced the TRAIL-induced cleavage of Bid. *β*-actin was used as a loading control

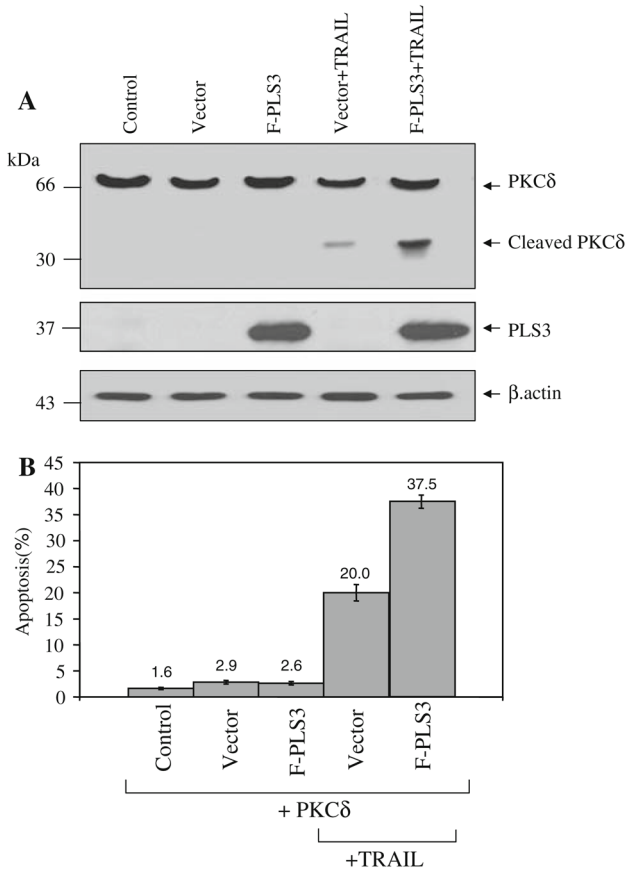

Fig. 6.

PLS3 enhances TRAIL-dependent PKC*δ* cleavage and cell death. (**a**) Subconfluent cultures of parental Jurkat cells and PLCRS3 stably transfected Jurkat cells were treated with TRAIL for 6 h. PKC*δ* expression was assayed by immunoblot with anti-PKC*δ*. *β*-actin was used as a loading control. PLS3 expression greatly enhanced the TRAIL-dependent PKC*δ* cleavage. *β*-Actin was used as a loading control. (**b**) Flow cytometry was used to assess Sub G1 DNA content. The extent of apoptosis was approximately 38% in the PLS3 TRAIL-treated cells compared to 20% in parental cells

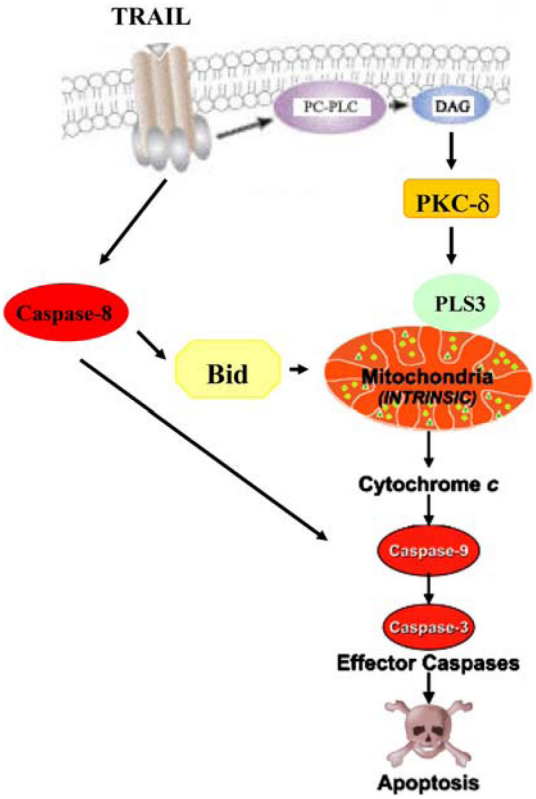

Fig. 7.
Scramblase 3 and TRAIL-induced apoptosis