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Review

The role of human phospholipid scramblases in apoptosis: An overview

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ABSTRACT

Human phospholipid scramblases (hPLSCRs) are a family of four homologous single pass transmembrane proteins (hPLSCR1-4) initially identified as the proteins responsible for Ca^{2+} mediated bidirectional phospholipid translocation in plasma membrane. Though *in-vitro* assays had provided evidence, the role of hPLSCRs in phospholipid translocation is still debated. Recent reports revealed a new class of proteins, TMEM16 and Xkr8 to exhibit scramblase activity challenging the function of hPLSCRs. Apart from phospholipid scrambling, numerous reports have emphasized the multifunctional roles of hPLSCRs in key cellular processes including tumorigenesis, antiviral defense, protein and DNA interactions, transcriptional regulation and apoptosis. In this review, the role of hPLSCRs in mediating cell death through phosphatidylserine exposure, interaction with death receptors, cardiolipin exposure, heavy metal and radiation induced apoptosis and pathological apoptosis followed by their involvement in cancer cells are discussed. This review aims to connect the multifunctional characteristics of hPLSCRs to their decisive involvement in apoptotic pathways.

1. Introduction

Phospholipids (PL) in the plasma membrane (PM) are asymmetrically distributed in such a way that choline containing PLs such as phosphatidylcholine (PC) and sphingomyelin dominate the outer leaflet, while the aminophospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are enriched in the inner leaflet of the lipid bilayer [1–4]. Externalization of aminophospholipids under stress conditions play a vital role in phagocytosis and apoptosis. PM asymmetry is maintained in a cell by a special family of proteins called PL translocators and are classified into two types namely ATP dependent and ATP independent PL translocators. ATP dependent PL translocators maintain PM asymmetry in normal cells at the expense of metabolic energy whereas ATP independent PL translocators include biogenic membrane flippases which mediates ATP independent PL translocation at a rapid pace in biogenic membranes [5]. ATP dependent PL translocators are further sub divided based on their mode of translocation and their specificity for PLs as flippases and floppases [5]. Flippases such as amino phospholipid translocators specifically transport amino PLs such as PS and PE from exoplasmic leaflet to the cytosolic leaflet. Floppases aid in exofacial transport of choline containing PLs such as PC [6–8]. Flippases and floppases together maintains asymmetry in the PM [9,10].

In 1996, Basse and co-workers isolated a 37-kDa integral membrane protein from human erythrocytes capable of energy independent scrambling of PLs in the presence of calcium [11]. Subsequent studies by the same group revealed that this protein is localized in the PM and is involved in the translocation of PL between the membrane leaflets when reconstituted into liposomes in the presence of Ca^{2+} and termed it as human phospholipid scramblase (hPLSCR1) [12]. Recombinant hPLSCR1 showed scramblase activity in artificial liposomes and also displayed Ca^{2+} [12] mediated surface PS exposure when overexpressed in Jurkat cells [13]. hPLSCRs were reported as multifunctional proteins because of its role in several key cellular processes such as cell proliferation [14,15], antiviral response [16–21], tumor suppression [22], protein interactions [23–27], transcriptional regulation [28–31] and apoptosis. This review primarily focuses on addressing the function of hPLSCRs in apoptosis and their deterministic role in different apoptotic pathways.

2. Human phospholipid scramblases

hPLSCRs are a group of type II single pass transmembrane proteins mediating Ca^{2+} induced ATP independent bidirectional trans-bilayer movement of PLs between the two leaflets of PM. Under normal conditions, scramblases are inactive but a thousand-fold increase in the intracellular Ca^{2+} level results in the activation of hPLSCRs along with the inactivation of flippases and floppases thereby disrupting the membrane asymmetry. In humans, scramblases constitute a family of four homologous proteins termed hPLSCR1-hPLSCR4. The predicted

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open reading frames encode proteins of 224 aa (hPLSCR2), 295 aa (hPLSCR3) and 329 aa (hPLSCR4) which are 59%, 47% and 46% identical to hPLSCR1 whereas hPLSCR5 is reported only in mRNA level. hPLSCR1, hPLSCR2, hPLSCR4 and hPLSCR5 are found to be closely clustered on chromosome 3 (3q23, 3q24), whereas hPLSCR3 is located on chromosome 17. Analysis of mRNA level expression of scramblases revealed that hPLSCR1 and hPLSCR3 are expressed in all tissues except in brain. Similarly hPLSCR4 is also expressed in variety of tissues except peripheral blood lymphocytes whereas the expression of hPLSCR2 is restricted only to testis [32]. Within a cell, hPLSCR1 and 4 was found to be localized to PM, cytoplasm and nucleus [26,33–35] whereas hPLSCR2 and hPLSCR3 was exclusively localized to nucleus and mitochondria respectively [34,36]. The differential gene expression in specific tissues needs further investigation as it is unclear and might have unique functions.

Multiple sequence alignment of hPLSCRs revealed several domains conserved from *C. elegans* to humans. Major domains of PLSCRs include (i) a putative EF-hand like calcium binding motif responsible for Ca^{2+} binding and activation [37,38], (ii) cysteine palmitoylation motif that helps in membrane anchoring [39], (iii) DNA binding motif that is involved in protein-DNA interactions during transcriptional regulation [31], (iv) a non-classical nuclear localization signal that aids in nuclear localization [26], (v) C-terminal helix that is essential for membrane insertion and scramblase activity [40], (vi) a cholesterol binding domain [41] and (vii) a proline rich domain (PRD) responsible for oligomerization mediated scramblase activity [34] (for a detailed review of the domain architecture, please refer [8]). Fig. 1 shows the multiple sequence alignment of hPLSCR1-4 from humans, mouse and *C. elegans*. While calcium binding motif, DNA binding motif and C-terminal helix were found to be highly conserved, PRD was not conserved among the homologs and across the species. Though hPLSCRs were initially identified as type II single pass transmembrane proteins, a recent report by Herate and co-workers revealed that overexpression of hPLSCR1 at the cell surface of differentiated macrophages resulted in the modification of the membrane topology of hPLSCR1 explaining that the protein may also adopt a type I transmembrane orientation exposing N-terminal region at the cell surface using the same transmembrane domain [42]. The physiological relevance for this flexible membrane topology is yet to be ascertained.

3. Is hPLSCR1 a true scramblase?

hPLSCRs were initially thought to be the proteins involved in Scott Syndrome, a bleeding disorder characterized by impaired PS exposure in platelets [43,44]. Subsequently, orthologs of hPLSCRs in different organisms were identified and characterized. hPLSCR1 was identified as the protein responsible for PL scrambling activity in erythrocytes and platelets [11]. Purified recombinant hPLSCR1 retained the PL scrambling activity *in vitro* when reconstituted to artificial liposomes [12]. Initial reports claimed the importance of hPLSCRs for elevated PS exposure levels on the cell surface during apoptosis. Zhao and coworkers reported that Raji cells stably transfected with hPLSCR1 cDNA showed higher PS exposure levels compared to untransfected cells [13]. However, subsequent research questioned the role of hPLSCR1 in PS exposure. Transcriptional activation of hPLSCR1 by interferons did not affect the PS exposure and increased expression of hPLSCR1 did not increase the PS exposure in several cell lines [45]. PLSCR1 deficient mice showed altered granulocyte production when treated with growth factors and impaired antiviral response to interferon but did not show any defects on PL scrambling. PLSCR3-null mice and PLSCR1 and PLSCR3 double mutant mice showed dyslipidemia and insulin resistance but did not alter PL scrambling [14]. Cells from PLSCR deficient *Drosophila* exhibited enhanced neurotransmitter secretion at larval neuromuscular synapses but did not show any alterations in lipid scrambling [46].

In 2010, Suzuki and co-workers identified TMEM16F, a member of calcium channels called anoctamins was directly responsible for Ca^{2+} dependent PS exposure in erythrocytes and a mutation in TMEM16F was responsible for Scott Syndrome [47,48]. A recent study identified that Xk-family protein Xkr8 exhibited PL scrambling activity and externalized PS in response to apoptotic stimuli and Xkr8 deficient cells did not exhibit apoptotic PS exposure [49–51]. These reports along with other key factors such as single transmembrane domain and smaller molecular weight compared to other transmembrane proteins questioned the PL scrambling role of PLSCRs.

Contradictorily, recombinant purified hPLSCR1, hPLSCR3 and hPLSCR4 showed scrambling activity *in-vitro* when reconstituted in proteoliposomes. Overexpression of PLSCR ortholog in *C. elegans* exhibited enhanced externalization of PS and apoptosis. PLSCR expression levels in *Dugesia japonica* (djPLSCR) was high when the planarians were stimulated with pathogen-associated molecular patterns suggesting that djPLSCR could play key roles in pathogen defense and apoptosis. Based on the reports, there might be two possibilities: i) hPLSCRs could be one among the many scramblases present in the cell and are triggered specifically at certain conditions, ii) Apart from scrambling activity, hPLSCRs could have several other key functions in the cell. Even after two decades of research revealing promising evidence for the various roles played by hPLSCRs, their exact function in a cell remains unclear.

4. Apoptosis and hPLSCRs

Apoptosis or programmed cell death involves a series of events resulting in regulated destruction and elimination of the targeted cell [52] and is essential for normal development, aging and maintenance of homeostasis by controlling cell populations in tissues. Apoptotic cells exhibit biochemical modifications such as protein cleavage, DNA breakdown and phagocytic recognition [53–55]. Caspases and metal dependent endonucleases mediate protein cleavage and DNA degradation respectively. Phagocytic recognition happens through the expression of cell surface markers *i.e.* exposure of PS, an anionic PL from the inner leaflet to the outer leaflet of the PM [56]. The major pathways leading to apoptosis of the cell include the extrinsic pathway and the intrinsic pathway, all of which converges to effector or execution pathway. Activation of caspases, cytochrome C release, DNA fragmentation and externalization of PS followed by phagocytosis are considered to be the hallmarks of apoptosis.

hPLSCR1 and hPLSCR3 are the most extensively studied proteins in the scramblase family and are reported to have crucial roles in apoptosis. During apoptosis, cells overexpressing hPLSCR1 was shown to have enhanced PS exposure, a hallmark of apoptosis. Upon UV treatment, hPLSCR1 stably transfected CHO-K1 cells and hPLSCR3 overexpressed cells displayed increased PS exposure and increased levels of caspase 3 and cytochrome C release thereby leading to apoptosis. hPLSCR1 plays a significant role in interferon mediated antiviral defense and apoptosis upon viral infection through the JAK-STAT pathway. In this section, a brief overview of the different pathways of apoptosis followed by the role of hPLSCRs in each of the pathways were discussed.

4.1. hPLSCRs in extrinsic pathway

Extrinsic pathway is mainly triggered by tumor necrosis factor receptor superfamily (TNFRSF) of proteins present in the PM and are subsequently mediated by a group of proteases termed caspases. TNFRSF are a group of transmembrane proteins that are mainly characterized by a cysteine rich extracellular domain named as the death domain which binds to cytokines thereby mediating the apoptotic signal from the PM to several downstream apoptotic pathways within the cell [57,58]. The cytokines that trigger apoptosis mainly include tumor necrosis factor (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL), TNF-related weak inducer of apoptosis (TWEAK) and Fas ligand [58–63]. Caspases are a family of cysteine-aspartate proteases that

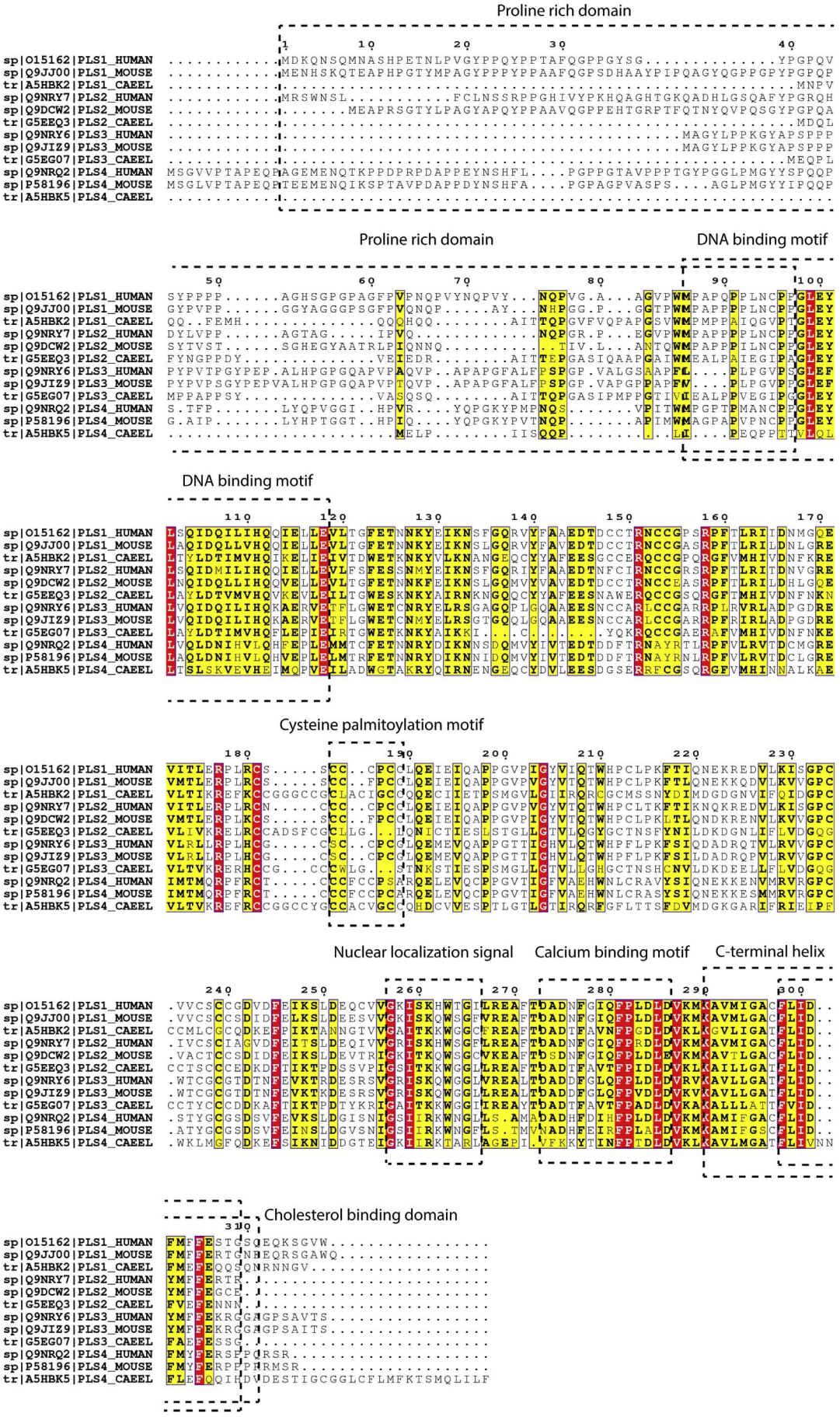

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Fig. 1. Multiple sequence alignment of hPLSCR homologs. Multiple sequence alignment was performed with CLUSTAL-Omega (EMBL-EBI) for the hPLSCR proteins (from top to bottom) are from *Homo sapiens* PLSCR1 (O15162), *Mus musculus* PLSCR1 (Q9JJ00), *Caenorhabditis elegans* (A5HBK2), *H. sapiens* PLSCR2 (Q9NRY7), *M. musculus* PLSCR2 (Q9DCW2), *C. elegans* PLSCR2 (G5EEQ3), *H. sapiens* PLSCR3 (Q9NRY6), *M. musculus* PLSCR3 (Q9JIZ9), *C. elegans* PLSCR3 (G5EG07), *H. sapiens* PLSCR4 (Q9NRQ2), *M. musculus* PLSCR4 (P58196) and *C. elegans* PLSCR4 (A5HBK5). Espript 3 was used for artwork of the sequence alignment.

are the main players in apoptosis. When the cell is marked for apoptosis, the cytokines are secreted by lymphocytes and macrophages. The trimeric form of the secreted ligands bind to the clustered receptors in the PM. The cytoplasmic death domain of the receptors then recruit adapter proteins which further interacts with procaspase-8 thereby forming the death-inducing signaling complex (DISC). Upon formation of DISC, procaspase-8 is cleaved to form the active caspase 8 which triggers several signaling pathways leading to apoptosis [56,64,65]. The main receptors that mediate extrinsic apoptosis include TNF receptor 1(TNFR1) and the Fas receptor. TNF-α binds to TNFR1 which recruits the adapter protein TRADD (Tumor necrosis factor receptor type 1-associated death domain protein) which further recruit initiator caspase procaspase-8 [66]. Procaspase-8 dimerizes and cleaves to form active caspase 8. Similar mechanism was followed for FasL/FasR cytokine-death receptor system [67]. Activated caspase 8 then triggers the cleavage of the protein Bid forming truncated Bid (tBid), which is an important signal for mitochondrial apoptosis and thus orchestrates the crosslink between intrinsic and extrinsic pathways [56].

hPLSCR3 was reported to actively participate in extrinsic apoptotic cascades mediated by TRAIL [36]. TRAIL induces apoptosis only in tumor cells but not in healthy cells thereby making it a potential therapeutic agent. TRAIL mediated activation of hPLSCR3 induced the activation of caspase 8 which cleaves Bid to form tBid. Meanwhile, Ca^{2+} efflux results from ER results in increase in concentration of Ca^{2+} in mitochondria thereby activating hPLSCR3 [68]. Activated hPLSCR3 transports CL and aids in the cytochrome C release thereby activating both extrinsic (Fig. 2) and intrinsic pathways (Fig. 3). Knocking down hPLSCR3 or employing the functional mutant of hPLSCR3 (F258V) failed to activate caspase 8, caspase 3 and other downstream targets, which strongly reinforces the involvement of hPLSCR3 in mediating apoptosis [36,69]. hPLSCR1 physically interacts with receptors expressed in lymphoid tissue (RELT) and co-localize to the internal compartments in HEK 293 cells. RELT is a member of TNF receptor superfamily that interacts specifically with TNF-receptor 1 associated factor 1(TRAF1) leading to apoptosis. RELT was overexpressed in hematologic tissues and was reported to induce apoptosis in HEK 293 cells. Oxidative stress response 1, a protein kinase was shown to phosphorylation and activation of hPLSCR1-RELT suggesting a possible connection of hPLSCR1 in TNF receptor signal transduction pathways and apoptosis [70]. Till date no reports suggest the involvement of other homologs in TRAIL induced apoptosis. hPLSCR1 and hPLSCR4 were also shown to be the receptors for secretory leucocyte protease inhibitor (SLPI) and CD4 receptor of the T-lymphocytes. SLPI is a serine protease inhibitor which is known to inhibit neutrophil apoptosis, whereas CD4 receptor is essential for HIV-induced apoptosis. Though the interaction was shown to have roles in antiviral intervention, its role in apoptosis remains to be identified.

4.2. hPLSCRs in intrinsic apoptotic pathway

Intrinsic apoptotic pathway is triggered by non-receptor mediated signals including endoplasmic reticulum (ER) stress, radiation stress, heavy metal toxicity, hypoxia, pathogenic infections and reactive oxygen species (ROS) [71]. While extrinsic pathway is mediated by death receptors and external stimuli, intrinsic pathway is solely driven by mitochondria and intracellular signals. The key event in the intrinsic pathway is the formation of mitochondrial permeability transition pore (MPTP) in the mitochondrial membrane which then releases several pro-apoptotic factors thereby triggering apoptosis [72]. The formation of MPTP is tightly regulated by Bcl family of proteins. In normal cells, Bcl-2 and Bcl-XI are localized to the mitochondrial membrane and inhibit the formation of MPTP by binding to proteins Bax and Bak. Dephosphorylation of the Bcl-2 associated death promoter (Bad) results in localization to mitochondria where it binds to Bcl-2 and prevents Bcl-2 mediated inhibition of Bax and Bak. As a cross-link between extrinsic and intrinsic pathway, caspase-8 which was activated by the DISC complex, cleaves Bid to form tBid. tBid binds to externalized CL and recruits Bax and Bak proteins. Bax and Bak in the membrane oligomerizes to form MPTP which aids in the release of pro-apoptotic factors such as cytochrome C and SMACs (second mitochondria-derived activator of caspases) [73]. Released cytochrome C binds to apoptotic protease activating factor – 1(Apaf-1) and ATP, which recruits procaspase-9 and forms the complex apoptosome [74,75]. Formation of apoptosome activates caspase-9 and triggers the effector pathway of apoptosis by activating effector caspases [76].

4.2.1. Heavy metals stress

Heavy metals such as arsenic, cadmium, chromium, lead and mercury are known to have dual roles: carcinogenicity and induction of apoptosis [77]. Heavy metal induced apoptosis could occur by several mechanisms including ROS production, caspase activation, p53 dependent and p53 independent pathways [78]. Arsenic is known to interact with SH groups of molecules such as glutathione which increases the accumulation of ROS thus leading to caspase activation and apoptosis [79]. Accumulated ROS disrupts the membrane potential of mitochondria thereby aiding the release of cytochrome C leading to activation of caspases [80]. Several reports have shown the effect of lead on apoptosis. Lead treatment triggered apoptosis in photoreceptor cells [81], hepatocytes [82] and macrophages [83]. Lead mimics calcium and stimulates calcium overload in mitochondria leading to decreased mitochondrial membrane potential, increased cytochrome C, caspase 3 and caspase 9 levels in rat rod photoreceptor cells [84]. Similar to lead, mercury induces apoptosis by mediating cytochrome C release through mitochondrial damage [85]. Eisele and coworkers revealed that exposure to mercury triggers the translocation of PS to the outer leaflet of cell membrane thereby resulting in apoptosis. The exact mechanism of PS exposure was not clear. The authors hypothesized the involvement of a PL scramblase that could be activated upon binding to heavy metals thereby inducing PS exposure [86]. Later, it was revealed that recombinant hPLSCR1 could bind to lead and mercury, similar to calcium and exhibit PL scrambling activity when reconstituted in synthetic liposomes. hPLSCR1 showed higher binding affinity to lead and mercury when compared to calcium [87]. Apart from these few reports, the mechanism of apoptosis induction by heavy metals and the role of hPLSCRs in the process remains unclear. There are no reports on the effect of heavy metals on homologs of hPLSCR1, especially hPLSCR3 as it has a major role in mitochondrial apoptosis. *In vitro* and *in vivo* studies to understand on the role of hPLSCRs in heavy metal induced apoptosis could shed more light on the mechanism and the pathways leading to apoptosis.

4.2.2. Radiation stress

The role of hPLSCR1 in UV induced apoptosis was first reported by Bailey and associates where in CHO-K1 cells lacking endogenous expression of hPLSCR1 were selected and treated with UV radiation. UV irradiated and hPLSCR1 stably transfected CHO-K1 exhibited increased PS exposure along with activation of caspase 3 thereby activating the intrinsic apoptotic cascades. Control CHO-K1 cells lacking hPLSCR1 did not have any significant changes in caspase expression levels. The other hallmarks of apoptosis like nuclear fragmentation, cell shrinkage and

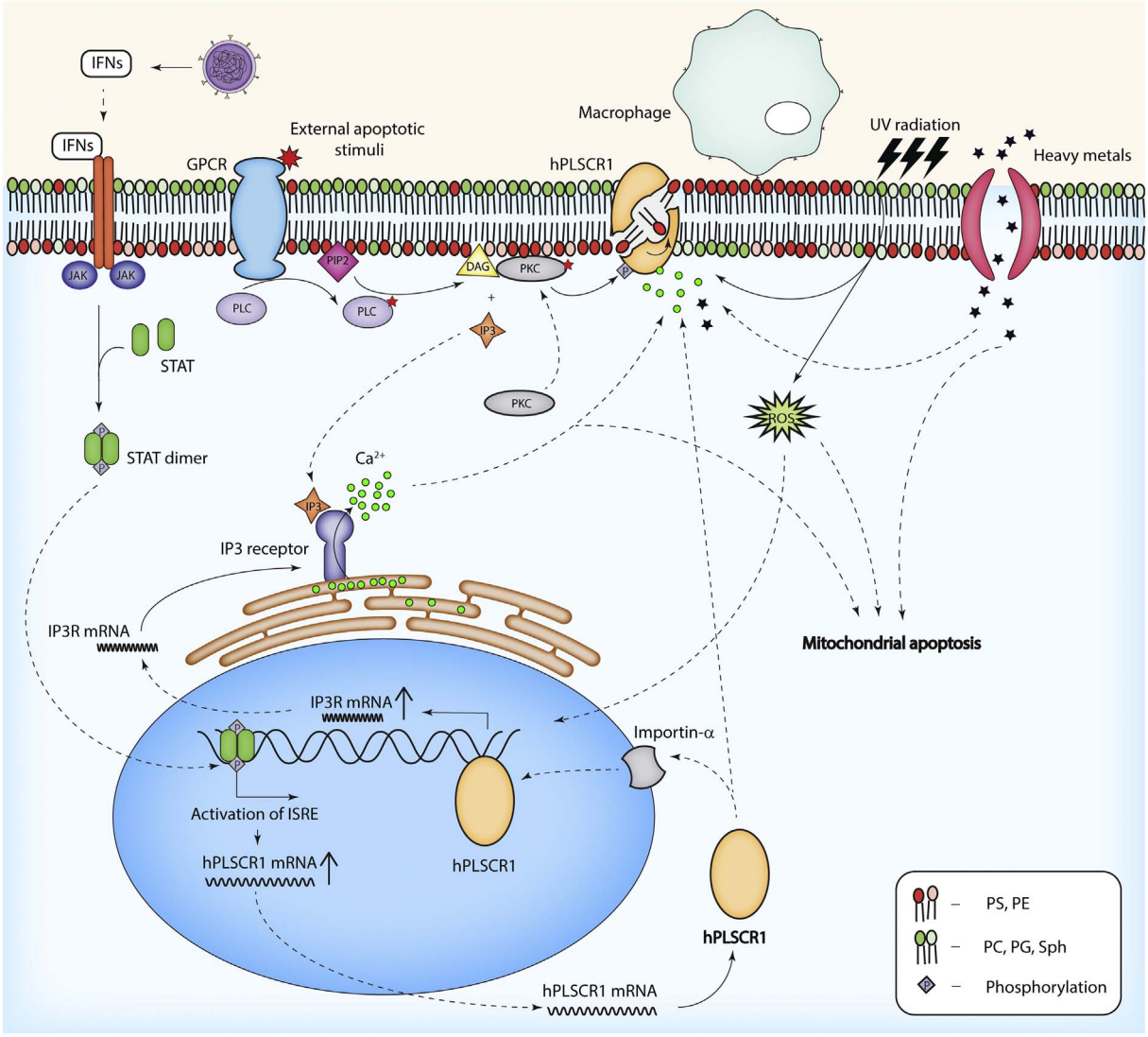

Fig. 2. Schematic representation of pathways leading to PS exposure from hPLSCR perspective. hPLSCR1 is overexpressed by IFNs through JAK-STAT pathway which then translocate to membrane and is phosphorylated by PKC-δ. ER induced stress increases the intracellular calcium concentration by 1000-fold which activates hPLSCR1. Activated hPLSCR1 externalizes PS, a hallmark signal for phagocytic engulfment of macrophages.

activation of pro-apoptotic factors were observed in hPLSCR1 containing CHO-K1 cell lines. These findings clearly suggest potential pro-apoptotic role of hPLSCR1 in UV induced apoptosis [88]. However, no such effects were pronounced in hPLSCR2 stably transfected CHO-K1 cell lines indicating active roles of hPLSCR1 but not hPLSCR2 in intrinsic apoptotic cascades [89]. The activation of intrinsic apoptotic pathway under UV irradiation by hPLSCR1 is independent of PKC δ activation as no change was observed in cells transfected with wild type hPLSCR1 and PKC δ phosphorylation site mutant hPLSCR1 [88]. hPLSCR3 on the other hand was identified to be a direct substrate of PKC δ phosphorylation, as inhibition of PKC δ by rottlerin drastically decreased the apoptotic potential [88,90]. Phosphorylation of hPLSCR3 noticeably increased under UV irradiation thus confirming the crucial role of PKC δ induced activation of hPLSCR3 [91]. The CL transport rapidly increased in the cells overexpressing hPLSCR3 under UV irradiation and the exposed CL helps in tBid recruitment and activation of Bax resulting in apoptotic cascades [92,93]. Reports also suggest that hPLSCR3 plays an important role in TNF-α induced apoptosis. The regulation of TNF-α induced apoptosis by hPLSCR3 was reported in HeLa cells. TNF-α induced the activation of caspase 8 by the formation of death domain and cleavage of procaspase 8. Bid was cleaved by caspase 8 to form tBid which was a key signal for activation of intrinsic pathway. An increase in intracellular Ca^{2+} levels activated hPLSCR3 thus leading to CL exposure to outer mitochondrial membrane. The exposed CL attracted tBid and aided in the release of cytochrome C thereby activating the effector pathway mediated by caspase 3 [69] (Fig. 3).

4.3. hPLSCRs in effector pathway

Both extrinsic and intrinsic pathways converge at a point leading to the effector pathway, the final stage in apoptosis. Effector pathway is characterized by activation of effector caspases such as caspase-3, caspase-6 and caspase-7 which acts upon several substrates in the cell thereby triggering cell death [94,95]. Caspase-3 is the most widely characterized member among the effector caspases and is activated by caspases from both extrinsic (caspase-8, caspase-10) and intrinsic pathways (caspase-9). Caspase-3 triggers the activation of endonuclease CAD that degrades nuclear DNA and is responsible for chromosomal condensation [96,97]. Externalization of the PS and phagocytosis of the apoptotic cells is the final major signal for cell death. During apoptosis, increase in Ca^{2+} concentration by several fold inactivates flippases and floppases and triggers hPLSCR1 to scramble the lipids in the PM. Caspases are also known to play a role in externalization of PS in

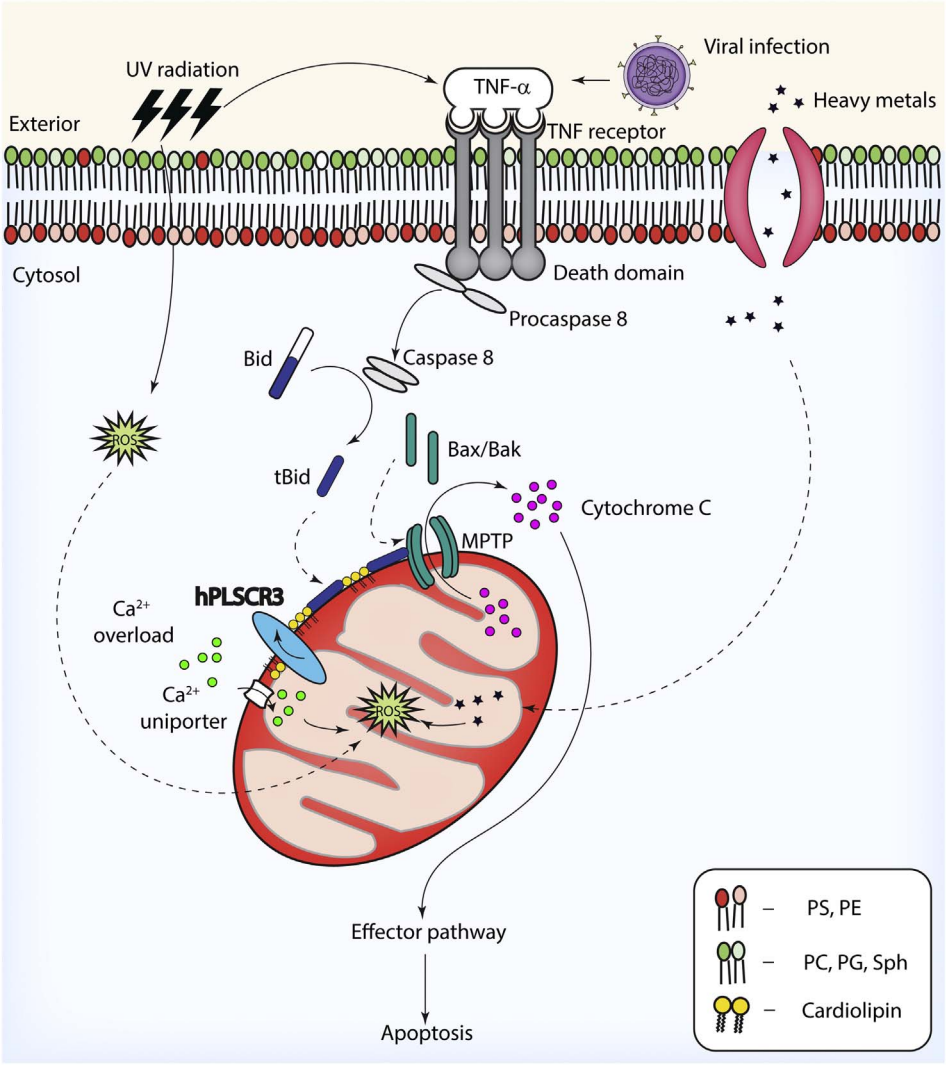

Fig. 3. Schematic representation of mitochondrial apoptosis. The major apoptotic pathways include extrinsic pathway by TNFR mediated death domain formation and caspase-8 activation. Activated caspase-8 cleaves Bid to form t-Bid which is the main trigger for mitochondrial apoptosis. Heavy metal induced stress and hypoxia results in ROS production which could aid in cytochrome C production. In normal cells, Bcl-2 and Bcl-XL proteins are bound to Bax and Bak and prevent their oligomerization. Upon induction of apoptosis, the BH3-only proteins such as Bad, binds to Bcl-2 and Bcl-XL thereby inhibiting the interaction of Bcl-2 to Bax and Bak proteins. ER stress mediated increase in Ca^{2+} concentration activates hPLSCR3 which flips CL to the outer membrane of mitochondria. tBid recruits Bax/Bak proteins thus aiding in cytochrome C release to the cytoplasm, a hallmark of apoptosis.

erythrocytes upon oxidative stress. PS exposure in apoptotic cells and the cellular fragments is considered to be the hall mark of apoptosis [56]. The exposure of PS on the outer leaflet aids non-inflammatory phagocytic recognition, thereby facilitating the uptake and disposal of apoptotic cells [98].

Protein kinase C – δ (PKC δ) is one of the many downstream targets of intrinsic apoptotic pathway. The effector caspase 3 activates PKC δ by cleaving the catalytic domain which further localizes to PM and phosphorylates hPLSCR1 at T^{161} [99]. It was evident from the study that the activation of hPLSCR1 during apoptosis is a result of PKC phosphorylation. One of the major apoptotic signals conserved from *C. elegans* to humans is the exposure of anionic PLs such as PS from the inner leaflet to the outer leaflet of the PM [100]. PS exposure is a signal for phagocytosis by macrophages that recognizes PS in the cell surface and engulf the apoptotic cells. hPLSCRs are long known to be involved in PS exposure by Ca^{2+} dependent externalization of PS to the cell surface. Though the role of hPLSCRs in externalization of PS is still debated, several reports reveal evidence for PL scrambling by hPLSCRs. Recombinant hPLSCR1 and hPLSCR4 has been shown to have Ca^{2+} dependent lipid scrambling activity when reconstituted in artificial liposomes [101,102]. Earlier in a report, we showed that hPLSCR1 oligomerizes in presence of Ca^{2+} and exhibited PL scrambling activity *in-vitro* and PRD of hPLSCR1 was essential for the oligomerization [34]. PRD of hPLSCR1 might function as a regulatory switch for controlling the PL scrambling function of hPLSCR1 during stress conditions such as apoptosis which should be further investigated.

4.4. hPLSCRs in pathological apoptosis

During viral infection, the host cell has an arsenal of antiviral defense mechanisms by which it tries to prevent the viral replication inside the cell. Several reports showed that apoptosis is one of the key processes undergone by the host cell as a part of antiviral defense [103]. The antiviral defense takes place at two stages: (i) primary defense by natural antibodies that prevent the invading virus from infecting the host [104] and (ii) by cytotoxic lymphocytes that kills the infected cells [105,106]. Upon viral invasion, the secondary defense by cytokines such as interferons are triggered which result in the stimulation of cytotoxic T cells and several downstream genes necessary for executing a successful response to the viral infection [107,108]. In primary defense, natural killer (NK) cells recognize and process the infected cells by antibody dependent cell-mediated cytotoxicity [109]. NK cells selectively target the infected cells by binding to multiple histocompatibility complex class I (MHC) [110]. The Fc receptor (CD16) in the NK cells recognize host immunoglobins bound to viral antigens in the target cell. NK cells upon interaction with target cells, secrete proteins called perforins and granzymes. Perforins are pore forming cytolytic proteins that undergo Ca^{2+} dependent oligomerization during degranulation thereby forming pores in the membrane of the target cell. Granzymes are a group of serine proteases that trigger

apoptosis by activating caspases [111,112]. A recent report by Fan and associates revealed that colorectal cancer cell lines treated with antibody against N-terminal domain of hPLSCR1 showed decreased cell proliferation [113]. This antibody might be responsible for the activation of NK cells which ultimately lead to apoptosis of the cells thereby making hPLSCR1 as a potential therapeutic target for triggering apoptosis in cancer cells.

hPLSCR1 expression is strongly stimulated by interferons in a variety of cell lines where it is known to enhance the antiviral activity of interferons by further activating downstream target genes as a part of interferon mediated antiviral defense [18,114]. hPLSCR1 was shown to suppress the replication of vesicular stomatitis virus and encephalomyocarditis virus in Hey1B cells whereas knocking down hPLSCR1 using siRNA showed an increase in viral replication [18]. Furthermore, hPLSCR1 was also found to have antiviral activities against Hepatitis B virus, HIV-1, HTLV further explaining its potent antiviral properties [16,17,20,21].

Toll like receptors (TLRs) recognize foreign protein or nucleic acid and triggers antiviral response and apoptosis. TLR1, 2, 4, 5, 6, and 10 are localized to PM and recognize pathogenic proteins such as bacterial membrane proteins or viral capsid proteins. TLR3, 7, 8, and 9 are localized to endosomes where they recognize nucleic acids [115,116]. hPLSCR1 was also reported to interact with TLR9 in plasmacytoid dendritic cells and enhance MyD880 dependent type I interferon responses. Knockdown of hPLSCR1 expression led to 60% decrease in Interferon (IFN) responses upon CpG-oligodeoxynucleotide (CpG-ODN) treatment [19]. In a separate study, CpG-ODN exposure to TLR9 transfected fibroblasts lead to the activation of Bax and caspase-3 resulting in apoptosis [117].

Transfection of dsDNA in immortalized normal ovarian surface epithelial cells lead to an increase in expression of endogenous hPLSCR1. hPLSCR1 upregulation resulted in the activation of proteins involved in the TLR signaling cascade. Interestingly, no changes in hPLSCR1 levels are observed in ovarian carcinoma cell lines and the reason remains unclear [118]. Degradation of foreign DNA is one of the main processes in antiviral defense. The invading pathogen releases DNA/RNA in the host cell which leads to interferon mediated stimulation of several nucleases that act and degrade the DNA [119,120]. DNA degradation is also one of the key processes in apoptosis and several apoptotic nucleases have been reported [121–124]. Apoptotic nucleases fall under two categories: cell autonomous nucleases and waste management nucleases. Cell autonomous nucleases are present in the nucleus and cleave DNA within the cell upon induction of apoptosis. Waste management nucleases digest non-nucleosomal DNA and are usually present in endoplasmic reticulum, lysosomes or secreted into extracellular space [119]. hPLSCR1 has been recently reported to have a novel non-specific nuclease activity to both DNA and RNA [125]. Nuclease assay with amino-acid modifying reagents revealed that histidine residues were critical for nuclease activity. Further studies are required to understand the mechanism of nuclease activity and the physiological role in apoptosis. Since, hPLSCR1 is stimulated by interferons and play a vital role in apoptosis, the nuclease activity of hPLSCR1 might have significance in antiviral defense mediated apoptosis which should be experimentally investigated.

5. Transcriptional regulation of hPLSCRs

hPLSCR1 is the most extensively studied homolog of scramblase family and is reported to have a number of interacting partners contributing to its multi-functional roles. Since hPLSCRs are involved in several key processes in the cell, understanding the regulation of expression of hPLSCRs is essential. hPLSCR1 was transcriptionally upregulated by IFNs during antiviral response which further activates several downstream targets of IFNs thereby enhancing the interferon mediated antiviral response. Recent reports revealed that hPLSCRs were regulated by the transcription factors c-Myc and Snail.

c-Myc is a proto-oncogene which is known to play crucial roles in several cellular processes including cell proliferation, protein synthesis, cell adhesion and apoptosis. Almost 15% of all the cellular genes are transcriptionally controlled by c-Myc [126]. It was shown that hPLSCR1 was transcriptionally upregulated by c-Myc and the hPLSCR1 could be one of the many targets of c-Myc triggered apoptosis [30]. Li and coworkers revealed that hPLSCR1 interacts with onzin which is a negative target for c-Myc [127]. Onzin is a protein that is involved in cell proliferation and survival, playing an anti-apoptotic role by interacting with protein kinase B (Akt) and its substrate E3 ubiquitin-protein ligase (MDM2). Binding of Akt and MDM2 to onzin inhibits the expression of p53, thus promoting cell survival [127,128]. However, hPLSCR1 competes with Akt and MDM2 for binding to onzin. hPLSCR1-onzin interaction reverses the anti-apoptotic and proliferative phenotype of onzin. Interaction of hPLSCR1 with onzin downregulates the expression of onzin further impairing the interaction with Akt and Mdm2 which results in increased expression of p53 ultimately leading to apoptosis [127]. Hence, transcriptional upregulation of hPLSCR1, downregulation of onzin by c-Myc, hPLSCR1-onzin interaction and upregulation of p53 could be simultaneous processes that play a key role in c-Myc mediated apoptosis which needs to be elucidated.

Recent reports show that expression of hPLSCR1 and hPLSCR4 was downregulated by the transcription factor (TF) Snail. SNAI1, commonly referred as Snail is a zinc finger protein that acts as transcription factors and play a major role in regulation of epithelial to mesenchymal transition (EMT). Snail family of proteins promotes the repression of E-cadherin, an adhesion molecule which promotes EMT [129,130]. Preliminary bioinformatics studies reveal that several putative binding sites for Snail were predicted in the promoter of hPLSCR1 and hPLSCR4. EMSA, ChiP assay and knockdown studies confirmed the transcriptional downregulation of hPLSCR1 and hPLSCR4 by Snail [28,29]. Snail was known to suppress TGF-β induced apoptosis and trigger EMT in hepatocytes. In a physiological context, EMT and apoptosis are known to be mutually exclusive processes and downregulation of hPLSCR1 and hPLSCR4 by Snail could be one of the switches for Snail mediated repression of apoptosis and progression of EMT.

hPLSCR1 was known to function as a transcriptional factor controlling the expression of other genes. Though many reports exist on the indirect downstream activation of several interferon stimulated genes by hPLSCR1, only one study revealed a direct evidence for hPLSCRs as a transcription factor. The residues M^{86}-E^{118} in hPLSCR1 representing the DNA binding motif interacts with the promoter of inositol 1, 4, 5 triphosphate (IP3) receptor type 1 (IP3R1) and enhances its expression [31]. IP3R1 plays a role in calcium signaling during apoptosis. IP3-gated intracellular calcium release channels are widely expressed in most types of cells and serve as a common pathway for releasing intracellular calcium upon stimulation by a wide array of signals, including growth factors and stress conditions such as apoptosis [131].

6. Pro-apoptotic and anti-apoptotic roles of hPLSCRs in cancer cells

Over expression of hPLSCR1 was observed during granulocytic and monocytic differentiation of leukemic cells upon treatment with all trans retinoic acid (ATRA) and phorbol 12-myristate 13 acetate (PMA). Chen and associates proved that PKC δ directly interacts with hPLSCR1 and mediates this function, suggesting the role of hPLSCR1 in differentiation induced by ATRA and PMA [132]. Anti-leukemic roles of hPLSCR1 were reported in U397 cell lines with inducible expression of hPLSCR1 under the control of tetracycline. Upon induction of hPLSCR1, the growth of U397 cells was hindered and there was an increase in etoposide induced apoptosis along with differentiation of these leukemic cells to granulocyte cells [15]. These results provide evidence for the apoptosis mediated tumor suppressor roles of hPLSCR1.

Over expression of interferon stimulated genes (ISG) and their

resulting proteins is one possible reason contributing to drug resistance and tumor progression in a variety of cancers. Aromatase inhibitor (AI) resistant breast cancer cell lines MCF-7:5C and AI resistant tumors had elevated levels of hPLSCR1 and IFITM1 when compared with the normal MCF-7 breast cancer cell lines [133]. hPLSCR1 was already reported to be an interferon stimulated gene which gets activated by JAK-STAT signaling pathway [18,114]. Over expression of interferon α resulted in increased expression levels of hPLSCR1. Knocking down STAT resulted in decreased expression levels of hPLSCR1 and IFITM1 and promoted apoptosis. The expression levels of STAT-1 and hPLSCR1 significantly increased in AI resistant breast cancer cell lines. AI resistant cells are not susceptible to aromatase induced apoptosis but undergo apoptosis in presence of estradiol. The estradiol induced apoptosis requires the upregulation of many pro-inflammatory factors like IFNs and MCF 7:5C cell lines already reported to exhibit elevated expression levels of STAT-1, IFN and hPLSCR1. When they are elevated, hPLSCR1 and STAT-1 sensitizes AI resistant breast cancer cell lines to undergo estradiol induced apoptosis by suppressing anti-apoptotic factors like Bcl-2 and activating the pro-apoptotic factors like Bax, thereby promoting apoptosis [134]. These findings suggest the involvement of hPLSCRs in variety of apoptotic responses in tumor cells.

hPLSCR1 was also reported to have anti-apoptotic/tumorigenic properties in cancer cells. hPLSCR1 acts as a therapeutic agent in governing the tumorigenesis of colon cancer. The tumorigenesis potential of colorectal carcinoma cell lines was attenuated by blocking the N terminal of hPLSCR1 with a monoclonal antibody specific to this domain both *in-vitro* and *in-vivo* [113]. When the cells were treated with an antibody against N-terminal domain of hPLSCR1, expression levels of caspase 3, caspase 8 and caspase 9 were increased thereby leading to apoptosis. Blockade of caspases with specific inhibitors impaired the apoptotic potential of the antibody against N-terminal domain and hence promoted tumor progression. Hence hPLSCR1 enhances the tumor progression of CRC cells and the interaction of the antibody to hPLSCR1 hindered the tumorigenic property thereby inducing caspase mediated apoptosis.

7. Conclusions and perspectives

hPLSCRs are single pass type II membrane proteins that mediate the translocation of PLs within the two leaflets of the lipid bilayer. Among the several multifunctional roles, this review focused on the vital roles of hPLSCRs in apoptotic pathways. Based on the existing reports, the overall apoptotic signaling cascade mediated by hPLSCRs could be briefly explained as follows. (i) External apoptotic stimuli triggers IP3 receptor mediated Ca^{2+} overload which activates hPLSCR1, (ii) hPLSCR1 is phosphorylated by PKC-δ and externalizes PS in the PM which acts as a phagocytic signal for macrophages, (iii) During apoptosis, TNF-α binds to TRADD forming the death domain ultimately resulting in caspase 8 activation, (iv) Activated caspase 8 cleaves Bid to form tBid, (v) hPLSCR3 externalizes CL in the mitochondrial membrane and results in mitochondrial apoptotic cascade leading to the cytochrome C release, (vi) hPLSCR1 showed scrambling activity upon treatment with heavy metals and thus could play a role in heavy metal induced apoptosis, (vii) UV treatment in hPLSCR1 and hPLSCR3 overexpressed cells lead to enhanced PS exposure and activation of apoptotic caspases, (viii) Formation of ROS triggers Ca^{2+} overload which could activate hPLSCR3 in mitochondria ultimately resulting in cytochrome C release and apoptosis.

Though research on hPLSCRs is at its peak in the past decade, several key questions still need to be investigated. (i) Apart from a few hypotheses, the mechanism of PL scrambling by hPLSCRs is still unclear and not experimentally determined. How can a single pass transmembrane protein perform lipid scrambling? What are the critical regions involved? Are hPLSCRs true scramblases? (ii) The reasons for the unique subcellular localization of hPLSCRs are yet to be deciphered. hPLSCR2 is found explicitly in nucleus whereas hPLSCR3 is found only in mitochondria. Since hPLSCR2 does not have PL scrambling activity, what could be the role of hPLSCR2 in nucleus? Even though hPLSCR1 and hPLSCR3 showed high sequence similarity and have all conserved domains intact, hPLSCR1 is localized to PM and cytoplasm whereas hPLSCR3 is localized to mitochondria. What could be the unique sequence in hPLSCR3 that target the protein to mitochondria? (iii) hPLSCR2 was expressed only in testis and hPLSCR4 was reported to be the expressed in brain but their roles in the corresponding tissues are not yet investigated. What is the reason for the variable expression of hPLSCRs in tissues? (iv) What could be the physiological mechanism of heavy metal binding and ROS on PL scrambling *in-vivo*? (v) PRD of hPLSCR1 was shown to have key domains necessary for protein-protein interactions. Till date, very few reports exist on the interaction of hPLSCRs with proteins involved in apoptosis and also reported that hPLSCR1 was found to be upregulated during apoptosis. What are the other apoptotic proteins that interact with hPLSCRs? (vi) hPLSCR3 was hypothesized to be the protein involved in CL exposure in mitochondria. What is the role of hPLSCR3 on tBid recruitment to mitochondria and further recruitment of Bax/Bak proteins?

For the past two decades, research on the hPLSCRs mainly focused on hPLSCR1 followed by hPLSCR3, whereas the functions of hPLSCR2 and hPLSCR4 remains unexplored. Understanding the functional roles of hPLSCR2 and hPLSCR4 and the overall interconnected functions between the members of hPLSCRs in apoptotic pathways and determination of crystal structure of hPLSCRs will be result in great advancement in understanding the multifunctional roles of these proteins.

8. Analysis

CLUSTAL-Omega (EMBL-EBI) was used for multiple sequence alignment [135] and Espript 3.0 was used for visualizing conserved regions from the multiple sequence alignment [136]. Adobe Illustrator CC 2015 was used for drawing the schemes in Figs. 2 and 3.

Transparency document

The Transparency document associated with this article can be found, in online version.

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