



Phospholipase D and cancer metastasis: A focus on exosomes

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ABSTRACT

In mammals, phospholipase D (PLD) enzymes involve 6 isoforms, of which only three have established lipase activity to produce the signaling lipid phosphatidic acid (PA). This phospholipase activity has been postulated to contribute to cancer progression for over three decades now, but the exact mechanisms involved have yet to be uncovered. Indeed, using various models, an altered PLD activity has been proposed altogether to increase cell survival rate, promote angiogenesis, boost rapamycin resistance, and favor metastasis. Although for some part, the molecular pathways by which this increase in PA is pro-oncogenic are partially known, the pleiotropic functions of PA make it quite difficult to distinguish which among these simple signaling pathways is responsible for each of these PLD facets. In this review, we will describe an additional potential contribution of PA generated by PLD1 and PLD2 in the biogenesis, secretion, and uptake of exosomes. Those extracellular vesicles are now viewed as membrane vehicles that carry informative molecules able to modify the fate of receiving cells at distance from the original tumor to favor homing of metastasis. The perspectives for a better understanding of these complex role of PLDs will be discussed.

1. Introduction

The phospholipase D (PLD) enzyme family belongs to the phospholipases superfamily and are found in a broad range of organisms such as viruses, yeast, bacteria, animals, and plants (Frohman, 2015). Classical PLDs are defined by the presence of at least one conserved HKD (HxKxxxxD with H: Histidine; K: Lysine; D: Aspartic acid; x: any amino acid) motif in the catalytic site that acts on phosphodiester bonds found in a wide range of substrates (Jenkins and Frohman, 2005). After activation of receptor tyrosine kinases and G protein-coupled receptors, the primary substrate of mammalian PLDs is phosphatidylcholine, one of the most abundant components found in the lipid bilayer of the plasma membrane, to yield the second messenger phosphatidic acid (PA) and choline (Tanguy et al., 2019a).

Most vertebrates have two classic PLD isoforms, PLD1 and PLD2, which share 50% homology at the amino acid level (Bowling et al., 2021). The general architecture of the HKD beta sandwich core excluding the flexible loops is conserved in human PLD1 and PLD2 (Bowling et al., 2021). Mammalian PLDs depend on the membrane-lipid phosphatidylinositol-(4,5)-biphosphate as a cofactor. PLD1 is activated by several protein effectors (Protein Kinase C, Ribosomal S6 kinase 2 (RSK2), and small G-proteins) both *in vitro* and

Abbreviations: ALIX, Apoptosis-linked gene 2-interacting protein X; Arf6, Adenosine diphosphate-ribosylation factor 6; ESCRT, Endosomal sorting complex required for transport; FIPI, Fluoro-2-indolyl des-chlorohalopemide; MAP1LC3, Microtubule-associated protein 1 light chain 3; MVB, Multivesicular bodies; PA, Phosphatidic acid; PEA-15, 15-kDa phosphoprotein enriched in astrocytes; PLD, Phospholipase D; SNARE, Soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VEGF, Vascular endothelial growth factor.

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in vivo, whereas PLD2 is generally considered constitutively active. Surprisingly, however, lipidomic analysis from PLD knockout mice models indicated that PLD1 is providing more PLD activity than PLD2 (Tanguy et al., 2020). This difference in intrinsic activity is thought to be mediated by an additional flexible loop near the substrate tunnel entrance in PLD1 which allows regulation of the size of active site entrance (Bowling et al., 2021). Additional domains found in some PLD family members such as PH and PX domains can affect their membrane localization (Du et al., 2003; Stahelin et al., 2004, reviewed in Bowling et al., 2021). These two PLD isoforms are rather ubiquitous and found in most tissues and cell types, although some questions remain unclear about their distribution (Jenkins and Frohman, 2005). Intense controversies about the subcellular distribution of PLD1 and PLD2, especially in intracellular membrane compartments are not completely settled, but at present a consensus proposes that PLD2 is mostly found at the plasma membrane and in the Golgi complex, whereas PLD1 can cycle between various compartments of the endo-lysosomal system and transiently associate with the plasma membrane and the trans-Golgi network. In mammals, four additional PLDs have been identified more recently. Among those, PLD6/mitoPLD is found at the mitochondrial outer membrane. PLD6 encodes a single HKD motif, requiring dimerization for lipase activity, and uses ceramide as a substrate to produce PA. PLD6 regulates mitochondrial dynamics (Huang et al., 2011) and acts as a 3' RNA endonuclease to generate PIWI-interacting RNAs, a form of endogenous RNAi that are critical for spermatogenesis (Nishimasu et al., 2012). PLD6 also plays a role in the Myc-mediated AMP-activated protein kinase activation, which in turn inhibits

Table 1

Models and function in which PLD isoforms were described in this review to contribute to cancer, with the corresponding references. We apologize for the publications that were omitted to keep this review concise.

PLD isoform	Model	Function	Source
PLD1 and/or PLD2	MDA-MB-231 cells (Human breast adenocarcinoma)	Apoptosis suppression	Chen et al. (2003)
	MDA-MB-231 cells (Human breast adenocarcinoma)	Cell survival	Hui et al. (2006)
	MDA-MB-231 cells (Human breast adenocarcinoma)	Cell transformation	Zhong et al. (2003)
	MDA-MB-231 cells (Human breast adenocarcinoma)	Rapamycin resistance	Hui et al. (2005)
	MDA-MB-231 cells (Human breast adenocarcinoma)	Apoptosis suppression	Chen et al. (2005)
	786-O and RCC4 cells (Human renal adenocarcinoma)	Hypoxia resistance	Toschi et al. (2008)
	SNU-484 cells (Human gastric carcinoma)	Apoptosis suppression	Cho et al. (2008)
	MDA-MB-231 cells (Human breast adenocarcinoma)	Proliferation	Kang et al. (2009)
	LOX cells (Human melanoma)	Increased extracellular vesicle release	Muralidharan-Chari et al. (2009)
	MDA-MB-468 cells (Human breast adenocarcinoma)	EGF induced calcium signaling	Stricker et al. (2021)
PLD1	Rat-2 cells (Murine fibroblasts)	Cell transformation	Buchanan et al. (2005)
	HeLa cells (Human cervical adenocarcinoma)	Increases autophagy	Dall'Armi et al. (2010)
	Human retinal microvascular endothelial cells	Angiogenesis	Zhang et al. (2010)
	Xenografted B16F10 and LLC cells (Murine melanoma and carcinoma)	Metastasis and angiogenesis	Chen et al. (2012)
	iBMK cells (Immortalized murine kidney epithelia)	Cell transformation	Sulzmaier et al. (2012)
	Primary PLD1 KO mice macrophages	Blunted phagocytosis	Ali et al. (2013)
	Xenografted HEK cells (Immortalized human kidney)	Cell survival	Han et al. (2018)
	Saos-2 cells (Human osteosarcoma) and primary osteoblasts	Mineralization	Abdallah et al. (2019)
	U266 and H929 cells (Human myeloma)	Apoptosis suppression	Wang et al. (2020)
	4T1 cells (Murine breast adenocarcinoma)	Exosome secretion and targeting	Ghoroghi et al. (2021)
PLD2/PLD2	HeLa cells (Human cervical adenocarcinoma) and mouse embryonic fibroblasts	Lipid droplet accumulation	Hussain et al. (2021)
	H1355 cells (Human lung adenocarcinoma)	Enhanced proliferation autophagy and DNA repair	Chang et al. (2022)
	Primary mouse metastatic cancer cells	Lipid droplet accumulation	Zhang et al. (2022)
	RBL-2H3 cells (Murine leukemia)	Exosomes production	Laulagnier et al. (2004)
	Fas-resistant A20 cells (Mouse lymphoma)	Apoptosis suppression	Oh et al. (2007)
	Primary human thyroid cancer cells	Cell transformation	Kim et al. (2008)
	EL4 cells (Murine lymphoma)	Migration and adhesion via focal adhesion kinase activation	Knoepp et al. (2008)
	MTLn3 cells (Murine breast adenocarcinoma)	Cellular invasion	Henkels et al. (2011)
	TC-135 cells (Human Ewing sarcoma)	Cell transformation	Nozawa et al. (2005)
	MCF-7 cells (Human breast adenocarcinoma)	MVB budding	Ghossoub et al. (2014)
PLD3	22Rv1 cells (Human prostate carcinoma)	Cell differentiation	Borel et al. (2020)
	Human breast carcinoma patients	Expression level correlated with patient survival	Althubiti et al. (2014)
	Human osteosarcoma patients	Anti-PLD3 antibodies serve as a biomarker	Guo et al. (2021)
	Primary human pheochromocytoma	Expression level increased	Unpublished data
	LCaP and PC3 cells (Human prostate adenocarcinoma)	Apoptosis upregulation/migration, invasion, and metastasis repression	Liu et al. (2021)
	Noninvasive intraepithelial neoplasia biopsy	Severity correlated to expression level	Uleberg et al. (2014)
	T24 and Calu-1 cells (Human bladder and pulmonary carcinoma)	Cell survival	Shi et al. (2007)
	Primary human peripheral blood mononuclear cells	Osteoclast formation	Hsu et al. (2010)

the Yes-associated protein and the transcriptional coactivator with PDZ binding motif in the Hippo pathway (von Eyss et al., 2015). PLD3 and PLD4 are endoplasmic reticulum-associated proteins with no lipase activity detected to date but show 5' exonuclease activity that remove circulating single-stranded DNA, preventing activation of the microbial genetic sensor toll-like receptor 9 in inflammatory cytokine response (Gavin et al., 2018). PLD5 is another PLD family member with no catalytic activity identified but has been reported as an oncogene involved in prostate tumorigenesis (Liu et al., 2021).

PLD function has been studied in tremendous contexts using biochemical, cellular, and now physiological approaches. Potential roles for PLD in general or for PLD1/2 specifically have been reported in numerous physiological settings including various aspects of membrane trafficking in normal conditions (Ammar et al., 2013a; Tanguy et al., 2016), but also ones relevant to cancer such as survival signaling, control of cell polarity, Ras activation, and cell migration (reviewed in Zhang and Frohman, 2014). Elevated PLD activity, as well as overexpression were reported in a wide variety of cancers such as breast, colorectal, esophagus, gastric, lung, renal, and stomach (Gomez-Cambronero, 2014). Mitogenic and survival effects have been observed and are associated with an elevated expression of PLD. After summarizing the different contributions of the different PLDs in primary tumor development (see Table 1), we will highlight some of the latest observations that suggest that PLDs may also contribute to metastasis progression through their involvement in exosomes biogenesis and function.

2. The multiple implication of PLDs in cancer

The first observations suggesting that PLDs are involved in cancer progression are now more than 30 years old but most of these original studies were performed on cell lines. For instance, melanoma cell lines, but not primary melanocytes, have high levels of PLD activity. This effect is protein kinase C-, Rho- and phorbol ester-dependent, suggesting that in this context PLD1 supports cancer progression rather than PLD2 (Riebeling et al., 2003). Since then, it has been shown that expression levels of one or the other PLD isoforms are increased in most cancers. For instance, increased PLD2 expression and activity is observed in human colorectal cancer when compared to normal mucosa (Oshimoto et al., 2003). Screening of 97 colorectal carcinomas revealed increased but variable PLD2 levels with an obvious correlation between PLD2 expression and tumor size as well as with patient survival, suggesting that PLD2 might be a prognostic indicator of these cancers (Saito et al., 2007). PLD1 was also later shown to be involved in tumor progression in human colon cancer tissues (Kang et al., 2008). Intriguingly, in non-small cell lung cancer six single nucleotide polymorphisms were identified in the catalytic domain of PLD1 (Ahn et al., 2012). Similarly, a PLD2 gene polymorphism was shown to be prevalent in a Japanese case study where it was demonstrated that a missense C1814T mutation resulting in a Thr/Ile substitution is associated with colorectal cancer (Yamada et al., 2003). However, lipase activity of either PLD1 or PLD2 was apparently not affected by any of those polymorphisms.

2.1. Altered PLD activity increases cell survival rate

Cell viability experiments revealed that PLD prevents apoptosis and acts as a survival signal in many cell types such as in epithelial-like MDA-MB-231 cells (Zhong et al., 2003). The multiple mechanisms by which PLD-mediated survival signals are generated in cancer cells involve many possibilities: i) suppression of phosphoprotein 2A, which reduces its association with the eukaryotic translation initiation factor 4E-binding protein 1 and the S6 kinase thus favoring cell transformation (Hui et al., 2005), ii) rapamycin resistance through the competition of PA generated by PLDs with the mTOR complex (Chen et al., 2005), iii) stabilization of mutant p53 (Hui et al., 2006) or Myc (Chen et al., 2003), iv) regulation of hypoxia inducible factor 1a at the translation level (Toschi et al., 2008), v) inhibition of apoptosis of cancer cells (Cho et al., 2008), through the expression of anti-apoptotic proteins, such as B cell lymphoma 2 proteins (Oh et al., 2007). Of interest, inhibition of PLD1 activity potentiates the antitumor effects of bortezomib in multiple myeloma cells by inhibiting the mTOR/NF- κ B signal pathway (Wang et al., 2020).

Autophagy is another widely studied topic in oncogenesis as this process recycles cellular organelles, facilitating cell survival in conditions of nutrient starvation, radiotherapy, and treatment with certain cytotoxic drugs (Hanahan and Weinberg, 2011), although these functions can be pro- or anti-oncogenic depending on the type of tumor and setting (Schmukler et al., 2014). PLD1 ablation decreases starvation-induced expansion of microtubule-associated protein 1 light chain 3 (MAP1LC3)-positive compartments, demonstrating its implication in autophagy (Dall'Armi et al., 2010). However, the effects of altering PLD1 or PLD2 activity on autophagy during cancer progression remain to be investigated in greater detail.

Alkylating agents and radiation exposure are routinely used for their anti-cancer effects, but therapeutic resistance is commonly occurring through metabolic reprogramming. Interestingly, the glycolytic enzyme Aldolase A directly binds to PLD2 and suppresses its enzymatic activity, while PLD1 compensates for and enhances proliferation, repair, and antiapoptotic capabilities. At the same time, along with alkylating agents or radiation exposure, Aldolase A and PLD1 jointly support various aggressive cancer phenotypes and the metabolic reprogramming of lung cancer cells. Therefore, both Aldolase A and PLD1 have predictive value for the survival of lung cancer patients (Chang et al., 2022). Along the same line, PLD1 promotes lipid droplet accumulation to support resistance of metastatic cancer cells (Zhang et al., 2022) and in response to nutrient removal (Hussain et al., 2021). PA was recently shown to be produced at mitochondria-associated endoplasmic reticulum membrane subdomains, where the lipid transfer proteins ORP5 and ORP8 control the biogenesis of these primary organelles for energy storage (Guyard et al., 2022).

Although most of the work on PLD and cancer focused on PLD1/2, recent observations also suggest that other PLD isoforms could be upregulated in some cancers. For instance, viral infections such as SARS-CoV-2 infection increase the detection of autoantigens, often found in cancers, and PLD3 was recently found to be among the most interesting hits of autoantigens linked to COVID-19 disease (Wang et al., 2021). Accordingly, autoimmune anti-PLD3 antibody was proposed to serve as a prognostic biomarker for the assessment

of osteosarcoma clinical outcomes (Guo et al., 2021). Together with VAMP3 and syntaxin-4, two proteins associated with the secretory pathway, PLD3 was found to be a marker for senescence and its expression level correlated with survival rate in different tumors, especially in breast cancer (Althubiti et al., 2014). Finally, we have observed that PLD3 levels was increased by 3 fold in human pheochromocytomas, a tumor derived from adrenal neuroendocrine chromaffin cells that oversecrete catecholamine and neuropeptides (unpublished data). A recent study also revealed that miR-145-5p upregulated apoptosis and repressed migration, invasion, and metastasis of prostate cancer cells via direct PLD5 modulation (Liu et al., 2021), whereas PLD6 expression levels correlated with grades of cervical intraepithelial neoplasia lesions (Uleberg et al., 2014).

2.2. PLD is linked to cancer metastasis

In addition to being a survival signal, PLD activity elevation also provides migration cues in several cancers, such as bladder, breast, lung, and skin carcinoma (Zheng et al., 2006). In agreement with the strong evidence linking PLD activity to the secretory machinery (Bader and Vitale, 2009; Tanguy et al., 2020), elevated PLD is associated with MMP9 release in a MAPK- and NF- κ B-dependent pathway in an acidic environment, mimicking the tumor microenvironment (Kato et al., 2005). Furthermore, PLD2 activity is implicated in migration and invasion of lymphoma cells via focal adhesion kinase activation (Knoepp et al., 2008).

PLD signaling also directly regulates or interacts with many other known triggers of cancer progression (Roth and Frohman, 2018). For instance, fibroblasts lacking PLD1 activity can form tumors only if exogenous PA is provided, indicating that PLD1 is necessary for Ras-mediated transformation (Buchanan et al., 2005). Accordingly, the RalA/PI3K-dependent Ras activation in different types of cancer cells enhances PLD activity, which in turn provides survival signals (Shi et al., 2007). Increased PLD1-dependent Ras activation was suggested to be linked to the 15-kDa phosphoprotein enriched in astrocytes (PEA-15) expression, which promotes G1-to S-phase transition in epithelial cells. Inhibiting PLD1 or interfering with the binding of PLD1 to PEA-15 reduced the activation of Ras in tumorous epithelial cells (Sulzmaier et al., 2012). Crosstalk between PLD2 and PDGF-mediated signaling or the oncogenic kinase RET have been observed, respectively, in Ewing sarcoma cells (Nozawa et al., 2005) and in thyroid cancer cells (Kim et al., 2008). PLD2 has been reported to stimulate guanine nucleotide-exchange activity for Ras, Rac and Rho small GTPases, all implicated in cell migration (Gomez-Cambronero, 2014). Invasion of MTLn3 breast adenocarcinoma cells is dependent on PLD2 and Janus kinase 3 (Henkels et al., 2011). Furthermore, PLD1 regulates neurotransmitter release and neurite extension via phosphorylation by RSK2 (Ammar et al., 2013b; Zeniou-Meyer et al., 2008), which acts as a prooncogenic manner in many pathways and favors metastasis (Romeo et al., 2011), suggesting a possible interplay of RSK2-PLD1 in the secretion of trophic factors that contribute to metastasis. Interestingly, PLD activity is also altered in hepatitis C virus-induced carcinoma (Kim et al., 2004). Moreover, PLD1 has also been shown to regulate components of the Hippo pathway which could favor metastasis (Han et al., 2018). Furthermore, PA can inhibit the membrane translocation of tumor suppressors by binding neurofibromin 2 and prevent the formation of a complex with monopolar spindle-one-binder protein 1 in link with the Hippo pathway (Han et al., 2018).

After the extensive use of primary alcohols as non-selective inhibitors of PA synthesis (Zhang and Frohman, 2014), the identification nearly a decade ago of more selective and isoform-specific PLD1/2 inhibitors prompted much research. Although this has led to revisiting earlier observations, those PLD inhibitors interestingly showed a negative effect on tumor growth in mice (Gomez-Cambronero, 2014). In addition to a general reduction of tumorigenesis inhibition of PLD1 activity decreased the number of metastases and vascularization following melanoma or carcinoma cell xenograft in mice (Chen et al., 2012). Bones are common sites of secondary tumors. Interestingly, inhibiting PLD resulted in lower amounts of osteoclast formation induced by secreted factors of lung cancer metastatic cells, therefore diminishing resulting bone metastasis. This effect seems due to less protein kinase C activation and thereby stopping the phosphorylation of extracellular regulated signaling Ser/Thr kinases 1 and 2 (Hsu et al., 2010). PLD inhibitor Halopemide or PLD1-selective inhibitor, led to a decrease in mineralization in primary osteoblasts and Saos-2 cells and primary osteoblasts isolated from PLD1 KO mice were significantly less efficient in mineralization as compared with those isolated from wild-type or PLD2 KO mice (Abdallah et al., 2019). Newly designed isoform-selective PLD inhibitors also clearly reduce invasiveness in metastatic breast cancer models (Scott et al., 2009; Su et al., 2009) or EGF-induced calcium signaling in human breast cancer cells (Stricker et al., 2021). Similarly, inhibition of PLD1 and PLD2 by triptolide decreases cell proliferation in MDA-MD-231 cells (Kang et al., 2009). Administration of the inhibitor fluoro-2-indolyl des-chlorohalopemide (FIPI) into wild-type mice or knock-out of PLD1 led to a significant reduction of tumor metastasis (Su et al., 2009).

2.3. PLDs may control the tumor microenvironment by favoring angiogenesis and metastatic progression

PLD1 plays an important role in the tumor microenvironment, which aids in tumor angiogenesis and metastasis (Barisano and Frohman, 2020). An interesting finding was obtained through implantation of WT melanoma and lung tumors into mice lacking PLD1 as the xenografts grew slowly and exhibited virtually no tumor angiogenesis (Chen et al., 2012). This was traced to reduced signaling through the vascular endothelial growth factor (VEGF) receptor, altering neoangiogenesis as the tumor grew hypoxic. In vitro work indicated that aortic endothelial cells lacking PLD1 generated fewer microvessels in response to serum stimulation, failed to reorganize into vascular cords when replated onto extracellular matrix substrates and adhered poorly to fibronectin, vitronectin, and collagen, signifying poor activation of integrins. Altogether these finding suggested that PLD1 regulates the interaction of the vascular endothelial cells with the extracellular matrix. PLD1 has also been linked to hypoxia-induced, VEGF-stimulated pathological retinal angiogenesis via siRNA studies, although the signaling pathways involved may differ in detail (Zhang et al., 2010). Neovascularization of tumors often results in morphologically and functionally abnormal blood vessels with increased permeability. This vessel leakage is thought to prevent cytotoxic drugs from fully infusing into the core of malignant tumors. PLD1 KO but not PLD2 KO mice have reduced

vascular permeability resulting from reduced extracellular regulated kinases 1/2, p38-mitogen activated protein kinase, and Akt signaling downstream of VEGF receptor 2 stimulation (Chen et al., 2012). Taken together, these results suggest that VEGF signaling is impaired in the absence of PLD1, which leads to reduced vascular permeability, that could potentially normalize tumor blood vessels and improve chemotherapeutic delivery to pre-established and vascularized tumors. Metastasis efficiency is also dependent on interaction of the tumor cells with activated platelets, which shield them from damage while traveling through the vasculature and facilitate their anchoring to metastatic sites and intravasation into the adjacent interstitial tissue. Accordingly, Chen and collaborators found that tumor cells exhibited decreased binding to PLD1 KO platelets, suggesting at least a partial explanation for the reduction in

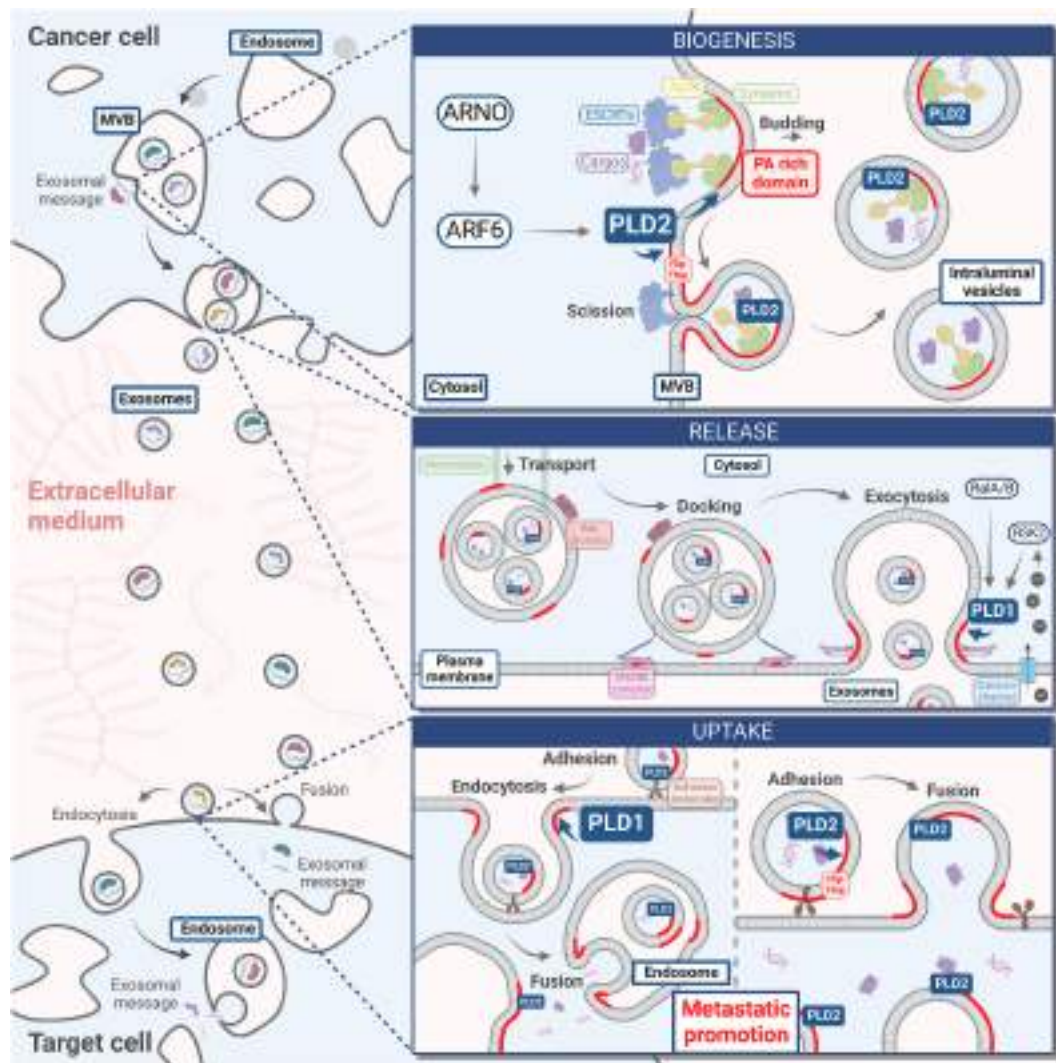


Fig. 1. Model depicting the pleiotropic functions of PLDs and PA in exosome biogenesis, release, and uptake during tumor metastasis. PLD may act on several steps of the exosomal cycle, from biogenesis and release from the primary tumor to uptake by the target cells. Increased PLD activity due to overexpression or mutations in the enzymes or their regulators leads to higher PA synthesis that promotes massive, abnormal, and dysregulated exosome release. The ARNO-Arf6 pathway activates PLD2 associated with endolysosomal membrane generating PA-rich domains and subsequently recruits the PA-binding protein syntenin, ALIX and the ESCRT proteins. Due to its cone-shaped structure, PA accumulation may also lead to an inward budding of the membrane during MVBs formation. Later, PA can also flip on the inner membrane leaflet of MVBs to favor membrane scission. Transport and docking of MVBs to the plasma membrane thanks to various actors of the cytoskeleton is also under the control of multiple GTPases. PLD1 regulated by RSK2 or RalA and RalB produces PA at the plasma membrane to generate negative membrane curvatures, facilitate priming of the SNARE complex and may then trigger release of exosomes. Although there is currently no evidence that PLD activity is directly implicated in exosome adhesion to target cells it seems, however, required for a proper incorporation of adhesion molecules during MVB biogenesis. PLD activity may also play a role in the two pathways leading to the exosome message transfer (i.e. direct fusion and/or endocytosis of the exosome with the target cell). On one hand, PLD1 activity in the recipient cells may stimulate endocytosis of exosomes. On the other hand, PLD2 accumulated inside exosomes could produce PA which can flop on outer membranes to induce their direct fusion with target cells. Of note, the PLD isoforms involved as described here may differ depending on the cell type.

metastatic seeding (Chen et al., 2012).

The contribution of the innate immune response to tumor development involves tumor-associated macrophages which can be either supportive or suppressive for tumor growth. This may be of importance here as both PLD1 and PLD2 have been implicated in the regulation of several steps of phagocytosis (Corrotte et al., 2006; Tanguy et al., 2019b). PLD1 KO macrophages exhibit several types of defects including blunted phagocytosis, spreading, and migration (Ali et al., 2013). Because local macrophages tend to be tumor-promoting and recruited ones tend to be tumor-suppressive, such migration defects in PLD1 KO mice may favor a shift in the balance toward tumor promotion and the establishment of secondary tumor sites.

3. PLD and exosomes in tumor development

The process of cancer is multifaceted and involves local reconfiguration to create favorable conditions to host circulating metastatic cells that have escaped the primary tumor. A relatively new and growing field is focused on exosomes released by tumor cells that can alter distant microenvironment and make it more hospitable for metastatic growth (Clancy et al., 2019), inhibit immune responses (Théry et al., 2009), promote drug resistance (Dong et al., 2020) and mediate angiogenesis (Aslan et al., 2019). Exosomes are 50–100 nm nanovesicles, marked by the tetraspanin CD63, that have emerged as a new intercellular communication system between an intracellular compartment of a donor cell towards the periphery or an internal compartment of a recipient cell. They are part of a larger family of secreted extracellular vesicles, which have been classified in various subtypes mostly depending on their size and on their membrane compartments of origin. Exosome release from cells appears to be a general biological process, as they have been reported in all biological fluids, but their contribution to cancer dissemination is now widely accepted. Nevertheless, the bioactivity of exosomes resides not only in their protein and RNA contents but also in their lipidic molecules. Hence exosomes are enriched in specific lipids and have been proposed to vectorize amphipathic molecules such as eicosanoids, fatty acids, and cholesterol (Pfrieger and Vitale, 2018). They also contain lipid related enzymes so that they can constitute an autonomous unit of production of various bioactive lipids. For example, lipidomic analysis showed that PA is substantially increased in exosomes secreted by PC-3 cells among other negatively charged lipids (Llorente et al., 2013). Pioneer work from the Michel Record group has shown that PLD1/2 isoforms are located on exosomes released by the leukemic cell line RBL-2H3 and that PA generated by PLD2 enhances exosome production after stimulation with ionomycin (Laulagnier et al., 2004). An association between PLD function and microvesicles released by tumors was later shown to be mediated by a mechanism involving the adenosine diphosphate-ribosylation factor 6 (Arf6) and PLD1 or PLD2 (Egea-Jimenez and Zimmermann, 2018; Muralidharan-Chari et al., 2009). Interestingly, Arf6 is an activator of PLD1/2 and has long been linked to it in the context of regulated exocytosis (Vitale et al., 2002). Indeed, the activation of Arf6 by the guanine nucleotide exchange factor ARNO on docked secretory vesicles represents a potent activator of PLD1 activity in neuroendocrine cells (Béglé et al., 2009; Vitale et al., 2002). The PA produced by PLD1 at this specific location acts a master regulator of secretion (Tanguy et al., 2020, 2021). Therefore, on the next part of this review we will focus on the molecular mechanisms of exosome biogenesis, release and uptake and discuss the contributions of PLD-generated PA during those processes (summarized in Fig. 1).

3.1. PLD and multivesicular bodies biogenesis

Exosomes typically originate from the fusion of endo-lysosomal structures called multivesicular bodies (MVB) with the plasma membrane (Tkach and Théry, 2016). However, it is important to note that other mechanisms for exosome generation, such as direct budding from the plasma membrane or plasma membrane-connected compartments have also been reported (Pegtel and Gould, 2019). MVB originate from Rab GTPases positive vesicles thanks to various pathways requiring lipid remodeling of endosomal membranes (Record et al., 2018). Among those different pathways that best described requires assembly of the endosomal sorting complexes required for transport (ESCRT) which enables clustering of cargo (ESCRT-0), membrane budding (ESCRT-1 and 2) and fission (ESCRT-3). Those proteins are recruited to endosomes thanks to syntenin through the intermediate of apoptosis-linked gene 2-interacting protein X (ALIX) (Friand et al., 2015). Interestingly, in a model of human mammary carcinoma cells (MCF-7), the depletion of Arf6 or its activator cytohesin-2 (also named ARNO) leads to a decrease in syntenin, ALIX and CD63 in exosomes, thereby affecting intraluminal budding from MVB. Investigation of Arf6 effectors implicated in this process pointed to a role of PLD2 in this model and excluded other Arf6 effectors such as PLD1, and phosphatidylinositol 4-phosphate 5-kinase alpha or gamma, enzymes producing phosphatidylinositol 4,5-bisphosphate (Ghossoub et al., 2014). This study also demonstrated the interaction between syntenin and PA via a PA-binding domain in its PDZ domain, underlining the importance of PLD-generated PA in MBV biogenesis. Indeed, an accumulation of this cone-shaped lipid generates membrane curvatures which are believed to favor membrane remodeling events (Kooijman et al., 2005), thus explaining its potential role in intraluminal vesicle budding. To summarize, the ARNO-Arf6 pathway activates PLD2 associated with endolysosomal membrane generating PA-rich domains and subsequently recruits the PA-binding protein syntenin, ALIX and the ESCRT proteins. Accumulation of conical-shaped PA may also lead to an inward budding of the membrane during MVBs formation. Later, PA can also flip on the inner membrane leaflet of MVBs to favor membrane scission (see Fig. 1: biogenesis).

It is also noteworthy that ALIX can bind the specific endo-lysosomal phospholipid lyso(bis)PA (also called Bis(Monoacylglycerol) Phosphate) by means of a convex surface in the Bro1 domain (Bissig et al., 2013). But it is important to keep in mind that despite the close chemical structure between LBPA and PA, no biosynthetic link between these two lipids has been identified to date.

3.2. PLD and exosomes release

Before being released, mature MVB need to be transported to the cell's periphery where they are docked to the plasma membrane. Multiple small Rab GTPases that regulate PLD activity have been implicated in these processes, depending on the cell type. On one side Rab27a and Rab27b silencing in HeLa B6H4 tumor cells induces, respectively, a size increase in MVBs or a redistribution of MVB away from the plasma membrane (Ostrowski et al., 2010). On another side, Rab11 seems to be implicated in the docking/priming of MVB to the cellular membrane in K562 cells in a calcium-dependent manner suggesting that MVB release is a form of regulated exocytosis (Savina et al., 2005). Interestingly, one of the best characterized function of PLD1 is its necessity in calcium-regulated exocytosis in neuroendocrine cells (Tanguy et al., 2020). Moreover, a dysregulation of regulated exocytosis pathway is a common characteristic of neuroendocrine tumors, explaining the excess of hormones that they secrete (Streit et al., 2022). Hypersecretion could therefore be a more general characteristic of tumoral cells explaining the overabundance of secreted exosomes by most type of cancerous cells. For example, numerous proteins involved in regulated exocytosis are overexpressed in human pheochromocytomas (Houy et al., 2022).

On another note, a recent report using isoform-selective PLD inhibitors on the aggressive 4T1 mammary tumor cells, mimicking human triple-negative breast cancer cells, identified a specific involvement of PLD1 in the RalA/B-mediated homeostasis of MVB and thereby tuning the biogenesis and secretion of a subtype of pro-metastatic exosomes (Ghoroghi et al., 2021). Of interest RalA or RalB silencing lead to a translocation of PLD1 from endosomal structures to the cytosol in nearly half of the cells without affecting the plasma membrane distribution of PLD2. However, either PLD1 or PLD2 specific inhibitors reduced by about 50% the number of secreted exosomes (Ghoroghi et al., 2021).

Regarding the fusion of MVB and plasma membranes step itself, it requires the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. For this, transmembrane proteins on MVBs (v-SNARE) and the plasma membrane (t-SNARE) assemble to drive the fusion of the two membrane and the release of exosomes. The v-SNARE VAMP3/cellubrevin and the t-SNAREs SNAP23 and syntaxin 4 have been directly implicated in exosome release (Kumar et al., 2020; Verweij et al., 2018). Interestingly, previous *in-vitro* studies showed that PA addition to SNAP23 vesicles enhances fusion (Vicogne et al., 2006), suggesting a central role for PA in MVB fusion with the plasma membrane (see Fig. 1: release).

3.3. PLD and exosomes uptake

Once released in the extracellular medium, exosomes need to bind to target cells membranes thanks to cells adhesion molecules on their external membranes. Among those, CD146 (also called MCAM) is of particular interest as it has been proposed to play a role in cancer progression (Wang and Yan, 2013). Interestingly, exosomes produced from RalA/B-depleted cells, hence having lower PLD activity, have also lower levels of CD146. This mechanism involving expression of PA and CD146 on exosomes favors metastasis by allowing exosomes targeting to the lungs (Ghoroghi et al., 2021). It is also noteworthy that the first phenotype described for PLD1 KO mice was an impaired signaling of the adhesion molecule integrins that prevented platelet aggregation (Elvers et al., 2010). All this suggest a role for PLD activity in exosome adhesion to target cells by impacting the presence of adhesion molecules on those extra-cellular vesicles.

After adhesion on target cells, exosomes can deliver their message through various endocytic mechanisms that have not been fully characterized yet (Mulcahy et al., 2014). Interestingly, a need for PLD activity has also been reported for various endocytosis pathways such as clathrin-mediated endocytosis (Antonescu et al., 2010), phagocytosis (Tanguy et al., 2019b), or compensatory endocytosis in secretory cells (Tanguy et al., 2021). Hence, contribution of PLD activity within target cells could directly promote exosome uptake and message delivery.

An alternative way of entry into target cells has also been proposed: fusion with the plasma membrane releasing the exosomal message in the cytoplasm (Mathieu et al., 2019). Although not much is known about this process, PLD2 has been reported to be found on secreted exosomes and to elicit a tumor microenvironment that promotes increased tumor stemness (Muñoz-Galván et al., 2019). Along the same line, prostate cancer-derived exosomes promote osteoblast differentiation and activity through PLD2 activity (Borel et al., 2020). Therefore, PLD enzymes captured during intraluminal endosomes vesicles budding can still produce PA on exosomal membranes and thus favor fusion with target cells (see Fig. 1: uptake). It is of note that the exosomal membrane leaflet to which PLD is associated has not been firmly established, so the model proposed is based on the most logical distribution assuming that PLD2 is associated to the cytosolic leaflet of MVBs.

4. Conclusion and perspectives

Taken together, it appears likely that PLD activity is involved in MVB biogenesis, exosomes secretion, targeting, as well as uptake although it is not clear whether PLD1 and PLD2 exhibit partially overlapping roles (redundancy) or specific phenotype. This is exacerbated by the fact that PLDs and their activators are often dysregulated in cancerous cells. Of interest a lipidomic analysis of exosomes secreted from RalA/B silenced cells revealed modification of the PA/phosphatidylcholine ratio, particularly for mono- and di-unsaturated lipid species (Ghoroghi et al., 2021), known to be PLD preferential targets (Tanguy et al., 2020). The exact role of PA on exosomal membrane is not clear but based on the multiple functions of PA in membrane remodeling (Tanguy et al., 2018), it is possible that PA is involved in one or several of the many modifications in membrane topology occurring during exosome biogenesis, secretion, targeting and cell entry.

Despite convergent evidence in hundreds of studies supporting the involvement of PLDs in various steps along the progression towards malignancy, as most of those were done using cell culture models, they are not always predictive of organismal responses. The

growing number of animal studies that have been performed in the recent years, however, suggest that most PLD isoforms could favor cancer and that both PLD1 and PLD2 play roles in tumor progression especially in the context of primary cancer cell migration, inflammatory responses, metabolic reprogramming, and distant microenvironment modification allowing metastatic growth. Combination of novel tools such as potent isoform-specific PLD inhibitors (May-Dracka et al., 2022) and PA sensors (Kassas et al., 2012; Potocký et al., 2014) with techniques such as advanced intravital imaging, *in vivo* cell tracking using MRI, single-photon emission computed tomography, and lipidomic imaging with increased resolution will advance our understanding of the role of PLDs in all the steps of tumor development and metastasis (Peralta et al., 2022). Mice lacking both PLD1 and PLD2 are viable, fertile, and overtly normal, and in agreement isoform-selective or dual PLD1/2 inhibitors appear well tolerated even for extended periods of time. Nonetheless, given the partial redundancy between PLD1 and PLD2 function and the several other means of generating PA in the cell as a form of induced compensation, critical roles for the PLD isoforms will need to be established empirically in each situation.

As highlighted in this review, PLDs and their product PA appear to contribute to optimal biogenesis and secretion of exosomes from primary cancer cells in preparation for the metastatic niche, but their exact subcellular localization in cells has not been well sorted out. The recent description of GFP knock-in models for both PLD1 and PLD2 (Barber et al., 2022), may help clarify the distribution of endogenous PLD, which likely are associated with several intracellular membrane compartments and may well cycle dynamically between them. As multiple species of PA generated by PLDs have been involved in a large extent of membrane trafficking events that require membrane remodeling steps such as membrane fusion or fission (Tanguy et al., 2016), these phospholipids might have pleiotropic roles in exosome formation and describing their specific functions in their biogenesis and release will be challenging. On this note, Tei and Baskin (2020) recently introduced an optogenetic PLD capable of quickly producing PA in specific membrane compartments such as the plasma membrane, endosomes, the trans-Golgi network and the endoplasmic reticulum. By using this approach, they established that specific PA production at the plasma membrane, but not in other compartments, can affect the Hippo pathway. Therefore, using similar approaches, it should be in theory possible to establish the specific roles of this phospholipid in the different steps of exosome secretion. It also remains to be investigated whether PA produced by PLDs on exosomal membrane also contributes to the entry of exosomes in target cells and to the delivery of the 'exosomal message'. Finally, using an up-to-date proteomic approach, Kattan et al. (2022) extended their original works on this key aspect and defined the protein interaction network for the human PLD family as well as for PA. This study revealed that diverse cellular signaling events involving this important lipid metabolic pathway are linked to PLD activity. Specifically applied to cancer models and to exosomal production, this type of approach will also help to get a better understanding of the contribution of PLDs and PA.

Disclosure of conflict

The Authors declare that they have no conflict of interest with publication of this manuscript.

CRediT authorship contribution statement

Alexander Wolf: draw the figure. **Stéphane Gasman:** Funding acquisition, wrote the manuscript and did the final editing, Funding acquisition, wrote the manuscript and did the final editing, All authors contributed and agreed to the final version of the manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

No data was used for the research described in the article.

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