**Phospholipase D and phosphatidic acid in the biogenesis and cargo loading of extracellular vesicles**

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**ABSTRACT**

Extracellular vesicles released by viable cells (exosomes and microvesicles) have emerged as important organelles supporting cell-cell communication. Because of their potential therapeutic significance, important efforts are being made towards characterizing the contents of these vesicles and the mechanisms that govern their biogenesis. It has been recently demonstrated that the lipid modifying enzyme phospholipase D2 (PLD2) is involved in exosome production and acts downstream of the small GTPase ARF6. This review aims to recapitulate our current knowledge of the role of PLD2 and its product phosphatidic acid (PA) in the biogenesis of exosomes and to propose hypotheses for further investigation of a possible central role of these molecules in the biology of these organelles.

**STRUCTURE AND FUNCTIONS OF PLDS**

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC), the most abundant membrane phospholipid, to generate phosphatidic acid (PA) and choline. There are 6 different mammalian proteins designated as PLDs (Figure 1).

Most of our knowledge about PLD biology refers to the PLD1 and PLD2 isoenzymes. PLDs participate in the normal maintenance of cellular membranes (1) and in a variety of physiological cellular functions, such as cell migration and proliferation, vesicle trafficking, cytoskeleton remodeling and morphogenesis (2-4). Most of these functions are attributed to PA generation. The catalytic domain of PLD1 and PLD2 appears to be under strict control, and a plethora of stimuli supports PLD activation.

Some proteins have been reported to regulate PLD activity through protein-protein interaction and/or phosphorylation/dephosphorylation processes. Regulators of PLD activity include tyrosine and serine/threonine kinases, and small GTPases, such as those of the ARF and Rho families (5, 6). Both ARF1 and ARF6 can activate human PLD2 (7, 8). For example, the activation of ARF6 by ARNO (ARF nucleotide-binding site opener), which is an ARF6-specific guanine-nucleotide exchange factor (GEF), results in increased activation of PLD and induces epithelial cell migration (9). Stimulation of chromaffin cells triggers the translocation of ARF6 from secretory granules to the plasma membrane and the concomitant activation of PLD at the plasma membrane (10). PLD activation can be blocked by addition of the ARF inhibitor Brefeldin A (11) and a synthetic myristoylated peptide corresponding to the N-terminal domain of ARF6 (10). It has also been reported that ARF6 can co-localize with PLD1 and PLD2 in membrane ruffles (12, 13). In spite of the
attempts of many groups to identify the PLD binding site for ARF (14, 15), to date evidence for direct PLD-ARF interaction is missing. PLD1 and PLD2 are known to differentially localize in the cell, possibly through specific post-translational modifications. PLD1 (16) and PLD2 (17) can be palmitoylated on two cysteine residues in their PH domain, facilitating the sorting of these isoforms into different compartments. At steady-state, PLD1 preferentially localizes to the perinuclear area, potentially associated with the endoplasmic reticulum, the Golgi apparatus, and/or late endosomes (18, 19). PLD2 is most often reported to be associated with the plasma membrane (6, 18), but also localizes in the cytosolic cell interior (12) and vesicular compartments (20). PLD2 has been shown to participate in the biogenesis of extracellular vesicles/exosomes, in two different cell types (21, 22).

EXTRACELLULAR VESICLES, SIGNALING ORGANELLES UNDER THE CONTROL OF PLD2

Extracellular vesicles (EVs) are limited by a lipid bilayer, and can be released by any type of cell. EVs have the same topology as the cell, and they contain cytosolic components and various types of nucleic acids. They also carry transmembrane proteins and lipids. In the last years, it became clear that EVs are true organelles, supporting cell-to-cell communication over short or long distance (23, 24). Moreover, the concept of ‘disease EVs’ was validated in various pathological contexts, like cancer, neurodegeneration and cardio-vascular disease (25). These ‘bad EVs’ can transport pathogenic molecules (including proteins, lipids and nucleic acids) and participate in disease progression. In cancer for example, ‘bad EVs’ promote carcinogenesis and tumor growth, angiogenesis, suppress immune response, mold the pre-metastatic niche and impair response to therapy. According to the current nomenclature, one distinguishes three types of EVs, namely exosomes, microvesicles and apoptotic bodies. Microvesicles originate from direct budding at the cell surface and their diameter can vary between 50 and 1000 nm. Apoptotic bodies result from cell fragmentation and can reach 5000 nm in diameter. Apoptosis is not concerned in this review. Exosomes find their origin in endosomal membranes. They originate from intraluminal vesicles (ILVs) that accumulate inside late endosomes and multivesicular bodies (MVB). MVB fusion with the plasma membrane allows ILV secretion in the extracellular environment, where these small vesicles are then designated as ‘exosomes’. Their size can vary from 40 to 150-200 nm. The term ‘exosome’ is abusively used in the literature. It often refers to the fraction of the secretome pelleting at 100.000 g (further referred to as ‘exosome-enriched EVs’ in this document). Yet, this fraction can be contaminated with small vesicles that are not of exosomal origin and complementary methods need to be used to prove the exosomal nature of EVs. Our understanding of the molecular mechanisms controlling exosome production is far from complete and some of these mechanisms appear to vary between cell types (26). Several molecules, including various specific Rabs, cortactin and SNAREs have been described to control the fusion of ‘secretory’ MVBs with the plasma membrane. The Endosomal Sorting Complex Required for Transport (ESCRT) was the first described mechanism for the formation of ILV and MVB (27, 28). It consists of four multimeric complexes (ESCRT-0, -I, -II and -III) and associated proteins (e.g., VPS4 and ALIX). ESCRTs are assembled in an orderly manner at the endosome. ESCRT-0, -I and -II recognize and sequester ubiquitinated membrane proteins, while the ESCRT-III complex is responsible for membrane budding and abscission to form ILVs (29, 30). Besides ESCRTs, neutral sphingomyelinase 2 (nSMase2) (31) and also syndecans, syntenin (32), heparanase (33), SRC (34) and ARF6 (21) were shown to participate in the budding of ILVs that will significantly contribute to the pool of exosomal vesicles (Figure 2).

In a model of human mammary carcinoma cells (MCF-7), the depletion of ARF6 or ARNO, an ARF6 GEF, leads to a decrease in several exosomal proteins such as syntenin, ALIX, CD63 and
syntenin and affects ILV budding (21). Investigation of the ARF6 effectors implicated in this process pointed to a role of PLD2 and excluded other ARF6 effectors such as PLD1, and also phosphatidylinositol 4-phosphate 5-kinase alpha or gamma (PIPSKα and γ), enzymes producing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) (21). Noteworthy, it has been proposed that PI(4,5)P₂, the product of PIPSKs, can activate PLD2, which leads to enhanced PA formation able to activate further PIPSks (12, 20). However, this does not seem to happen on the limiting membrane of MVBs, as the knock-down of PIPSks did not impact on syntenin intra-endosomal budding (21). These data are consistent with the observations that ARF is able to activate PLD in the absence of PI(4,5)P₂ (35). Basically, on endosomal membranes ARF6-PIPSK rather supports the recycling of syntenin and associated transmembrane protein cargo, pending on the coincident detection of peptide and PI(4,5)P₂ by the syntenin PDZ domains (see Figure 2 A-pathway) (36, 37). On the contrary, ARF6-PLD2 supports the budding of syntenin cargo-loaden ILVs inside the MVBs (see Figure 2 B-pathway) (21). This model is in the line of the observations of Laulagnier and coworkers (38) showing that stimulation of RBL-2H3 cells with ionomycin boosts the release of exosome-enriched EVs, in a PLD2-dependent manner. Indeed, ionomycin is an ionophore which induces the hydrolysis of PIP₂ and the release of calcium to the cytoplasm. One could speculate that PIP₂ hydrolysis would impair the recycling pathway and may indirectly favor the formation of ILVs. Moreover, high levels of intracytosolic calcium could facilitate the MVB-plasma membrane fusion events necessary for the secretion of ILVs as exosomes (39). Interestingly, syntenin also directly interacts with PA. While ARNO appears to be the preferred ARF6 GEF for inward budding (away from the cytosol, with formation of ILVs inside the MVB) (21) the stimulator of ARF6 that is supporting recycling pathways (necessitating endosomal outward budding, towards the cytosol) still needs to be identified. Clearly, syntenin-ALIX interaction and ESCRT are necessary for the exosomal pathway. As ESCRT was recently shown to also stimulate inward budding (40), it would be interesting to test for the impact of ALIX and ESCRT on the syntenin recycling pathway.

KEY ROLE FOR PA PRODUCTION IN EXOSOME BIOGENESIS?

PA (the product of PLD) represents the simplest phospholipid (Figure 3). Despite its simple structure and relatively low abundance (1-4% of the total of phospholipids (41, 42)), PA is important for membrane dynamics, i.e. fission and fusion (43). The function of PA is likely in part due to its ability to induce a negative membrane curvature because of its small headgroup (forming a ‘cone’ that might favor endosomal intraluminal budding). Moreover, PA can also directly interact with proteins (43, 44). Unfortunately, no consensus amino-acid sequence defines a PA-binding site.

PA enables an electrostatic/hydrogen bond switch (45) when engaging with protein ligands and the negative charge of the PA headgroup is increased from −1 to −2 and stabilized upon formation of hydrogen bonds with lysine and arginine residues of the interacting protein (46). As commonly observed for protein-lipid interactions, the membrane lipid environment and the nature of the acyl chains can influence the recognition of PA by interacting proteins (47).

PA is implicated, for instance, in regulated exocytosis, in several different cell lines, such as the secretion of von Willebrand factor from endothelial cells (48) and insulin from pancreatic β-cells (49). In RBL-2H3 cells, PA is controlling the trafficking of glycoconjugates from the Golgi to the plasma membrane (50). Whether PA might be essential for budding in endosomes and for MVB formation has not been directly investigated. One study indicates that PA is 1.8 fold enriched in exosome-
enriched EVs versus lysates from PC-3 cells. Yet PA accounts for only 0.16% of the total lipids in these organelles (51) and other studies failed to detect PA (21, 38, 52).

What might explain the discrete amount of PA in exosome-enriched EVs? The team of Michel Record observed that active phosphatidate phosphatase 1 (PAP1) is present in exosome-enriched EVs derived from RBL-2H3 cells (53). Upon the incubation of BODIPY-PA, 60% of the PA was hydrolyzed into diglycerides within 15 min. It might thus be that PA is rapidly degraded in these organelles. Alternatively, during ILV biogenesis PA might flip-flop and be restricted to the abscission sites, the membrane domains with the highest curvature.

Yet, PA might be key at several stages of exosome formation, because of its biophysical properties (Figure 4A-B) and/or due to direct interactions with effector proteins involved in the process of ILV budding (Figure 4C-E). Moreover PA could be important for the transport (Figure 4F) and plasma membrane docking (Figure 4G) of ILV-filled MVBs.

Because of its negative curvature, PA production could stimulate the initiation of the ILV budding (Figure 4A). Noteworthy, lipid-binding data suggest that syntenin can directly interact with PA (21). Irrespective of the curvature, syntenin interaction with PA could thus concentrate the syntenin/syndecan/CD63/ALIX complexes at the nascent bud, with ALIX-ESCRT further stimulating the budding process (Figure 4C). PA could also support ILV-budding independently of ESCRT. Indeed, neutral sphingomyelinase 2 (nSMase2) has also been proposed to interact with PA (54, 55) and this binding activity could support nSMase2 targeting (and ceramide formation) to MVBs (54). Interestingly, PA can interact with SRC through the SH4, unique lipid binding region (ULRB) and SH3 domains, stimulating its activation by favoring the open conformation (56). In turn, in cells like MCF-7, SRC can activate PLD2 by phosphorylating its tyrosine S11 (57). Such positive feedback loop could support sustained ILV production and would be totally consistent with the recently established role of SRC in exosome biogenesis (34). Whether the production of PA is required or not to initiate the feedback loop is unclear. Possibly the scaffolding function of PLD2 might be implicated. SRC might recruit PLD2 by direct interactions. For example, the SH2 domain of SRC could interact with the two phosphotyrosine motifs (P-YxN) localized in its PX domain as it was shown for Grb2 (58). Alternatively, the interaction could be indirect as the PH domain of PLD2 seems necessary for co-immunoprecipitation with SRC (59) (Figure 4D). Interestingly, there is evidence that RAS mediates the activation of PLD that is induced by SRC in vivo (60), but whether RAS might function in exosome biogenesis and cargo-loading by acting on the PLD2-PA axis is unexplored (Figure 4). Possibly after PLD2 action, a flip-flop of PA from the endosomal membrane facing the cytosol to the luminal membrane could help the completion of the ILV bud (Figure 4B). Indeed lipids such as PA can spontaneously move from one leaflet to the other within seconds or minutes (61).

In addition, annexins, a family of Ca\(^{2+}\)-regulated phospholipid-binding proteins that can interact with PA (62) could help the fission of ILVs (Figure 4E). Indeed, annexins have been proposed to be mediators or regulators of membrane fusion by inducing membrane curvature (63) and have been identified in extracellular vesicles enriched in exosomes (64) and localize at MVBs (65). The conversion of PC to PA mediated by PLD2 could even lower the Ca\(^{2+}\) requirements for membrane fusion in the presence of annexins (66). Signaling proteins such as ribosomal S6 kinases (S6K) are activated by PA and thereby support actin polymerization (67). Such actin polymerization when taking place at the MVB outer membrane could support the traffic of MVBs to the plasma membrane
(Figure 4F). Noteworthy, PA also binds to the tyrosine kinase Fer and enhances its ability to phosphorylate cortactin, a protein that promotes actin polymerization (68). Recently, cortactin has been described to act as a positive regulator of exosome secretion (69, 70). PA-production on the limiting membrane of MVBs could thus help exosome release in several ways (Figure 4G).

OTHER POSSIBLE ROLES FOR PLD2 IN EXOSOME BIOGENESIS?

PLD2 could also act in the biogenesis of bis(monoacylglycero)phosphate (BMP) (also called lysobisphosphatidic acid (LBPA) by cell biologists) by stimulating the production of its precursor phosphatidylglycerol (PG) (71). BMP concentration raises from 1 to 15% of total phospholipids between early and late endosomes in BHK cells (72). BMP has not been detected elsewhere in the cell and exhibits unique pH-dependent fusogenic properties (72, 73). BMP can induce the formation of multivesicular liposomes when their lumen is acidic and can interact with ALIX (73) and hsp70 (74) indicating that BMP can participate in exosome biogenesis (75).

PLD2 has been shown to function as a GEF for small GTPases, in particular RAC2 (76). It has been proposed that PDZ proteins might mediate the GEF activity of PLD2 (77). Whether PLD2 could work as a GEF for ARF6 and whether such activity might participate in exosome formation remains an open question.

Of course, direct PA-interacting proteins could also be targeted to exosomes upon PLD2 activation. Cytosolic proteins such as protein kinase C eta (PKCη), for example, accumulating in urinary exosomes (78) and interacting with PA (79), could be targeted to these vesicles due to PA interaction.

CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, PLD2 emerges as a regulator of exosome production. The role of PLD2 in the biogenesis of exosomes can be related to its enzymatic activity and the production of PA in various ways.

First, PA could stimulate the production of ILVs because it is a cone-shaped phospholipid that induces negative membrane curvature and that can favor membrane fusion and fission events. Furthermore, PA at the limiting membrane of MVBs could participate in the recognition and assembly of members of the ESCRT machinery because it directly interacts with syntenin that connects to ESCRT via ALIX. Additionally, PA might support a positive feedback loop including signaling proteins like SRC. Noteworthy, PA by its property to interact with a plethora of cytosolic proteins could also work as an anchor for their incorporation into exosomes. PA might thus not only affect the number but also the composition of exosomes.

In conclusion, PLD2 might integrate an important number of signaling pathways that participate in the ILV formation and/or the release of exosomes. Such mechanisms need now to be addressed. This new knowledge could pave the way for the development of new strategies to modulate exosome production and the biological effects of these vesicles.
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Figure 1. Domain structures of the mammalian PLDs. Indicated are the amino (N) and carboxyl (C) terminals. Domains shown are: in blue, the phox domain (PX); in green, the plekstrin-homology (PH) domain; in red, the catalytic sequence HxxxxxKxD/E (HKD domain); in orange, a transmembrane domain (TM); in purple, the PIP2 binding site; and in yellow, the PLD1 loop region. UniProt codes: PLD1 (P3393), PLD2 (Q8IV08), PLD3 (Q96BZ4), PLD4 (Q8N7P1) and PLD5 (Q8N2A8).

PLD1 (80) and PLD2 (18) compose the classical HKD subfamily and are about 50% identical in amino acid sequence (18). They contain 3 highly conserved HKD (HxxxxxKxD/E) domains necessary for their enzymatic activity; a plekstrin-homology (PH) domain which binds phosphoinositides and anionic lipids (80), and also serves as docking site for recruitment of SH2-domain containing proteins like the growth factor receptor bound protein 2 (Grb2) (58); a phosphoinositide binding phox domain (PX) involved in targeting proteins to cell membranes; and a putative polybasic PIP2-binding site, located between the catalytic HKD motifs (1, 15). The other 4 isoenzymes lack PX and TM domains and form a group of “non-classical PLDs”. PLD3 (81) and PLD4 (82) contain the canonical pair of HKD domains although to our knowledge no catalytic activity has been demonstrated for these isoenzymes. They are localized in the endoplasmic reticulum (ER) and are anchored by an N-terminal transmembrane domain and the catalytic domains are oriented in the ER lumen (83). PLD6 is anchored by an N-terminal transmembrane domain into the outer surface of mitochondria and hydrolyzes cardiolipin to generate PA (84). There is no publication yet concerning PLD5, but it shares similarities with PLD3 and PLD4 (85).
Figure 2. Mechanisms that control the recycling of syntenin cargo to the plasma membrane (A) or the sorting of cargo into ILVs (B). A. Syntenin-mediated endosomal recycling to the plasma membrane depends on the activation of ARF6 on perinuclear recycling endosomes, the recruitment/activation of the ARF6 effector PIP5K, and on the direct interaction of syntenin with PIP2 (37, 86). The molecular mechanism supporting syntenin-PIP2 dependent recycling is due to the direct interaction of syntenin with PIP2, and cargo, such as syndecans (37) or Frizzled 7 (36) receptors. B. ARF6 and PLD2 affect exosomes by controlling the budding of ILVs into MVBs (21). The loading of syntenin and of syntenin cargo, such as syndecans and CD63, in ILVs also depends on ARF6, but relies on its effector PLD2 (21), and probably its reaction product PA.
Figure 3. Phosphatidic acid (PA) and phosphatidylcholine (PC) structure. PA comprises a glycerol backbone, with two fatty acids bonded to carbons 1 and 2 forming the non-polar acyl chains of the lipid, and a phosphate group bonded to carbon 3, forming the negatively charged polar headgroup of PA. The carbon 3 is bonded to a choline group in PC giving a cylindrical shape to the molecule.
Figure 4. Model illustrating the possible roles of PA and PLD2 at the limiting membrane of MVBs to support ILV formation (A-E) or the targeting of MVBs at the plasma membrane (F-G). See text for more details.