Published in final edited form as:

Biochim Biophys Acta. 2009 September ; 1791(9): 845–849. doi:10.1016/j.bbalip.2009.05.011.

Phospholipase D in Endocytosis and Endosomal Recycling Pathways

Julie G. Donaldson

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

The discovery that Arf GTPases, mediators of membrane traffic, activate phospholipase D (PLD) raised the possibility that Arfs could facilitate membrane traffic by altering membrane lipid composition. PLD hydrolyzes phosphatidylcholine to generate phosphatidic acid (PA), a lipid that favors membranes with negative curvature and thus can facilitate both membrane fission and fusion. This review examines studies that have reported a role for PLD in endocytosis and membrane recycling from endocytic pathways.

Keywords

Arf6; endocytosis; recycling; membrane traffic; phospholipase D

1. Introduction

Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of phosphatidylcholine (PC) to form choline and phosphatidic acid (PA), a signaling lipid which can be further converted to diacylglycerol (DAG). For many years, interest in PLD centered on its function in signal transduction pathways and the ability of both growth factor and G protein-coupled receptors (GPCRs) to lead to activation of this enzyme [1,2]. It was later that cell biologists became interested in the ability of its product, PA, to facilitate membrane traffic, which is the movement of membrane and soluble proteins in vesicular carriers from one membrane compartment to another. Much of the work in the area of membrane trafficking has focused on the roles played by proteins in this process, the coat proteins, Arf and Rab GTP-binding proteins and SNAREs that facilitate vesicle formation, transport and fusion with the target organelle. The discovery reported in 1993 by two groups [3,4] that Arf proteins could activate PLD and thereby alter membrane lipid composition opened up new possible mechanisms for Arfs to mediate membrane traffic. At the time, Arf1 was known to associate with the Golgi complex and be required for the formation of coated vesicles that transport membrane and contents within the Golgi and ER-Golgi system [5]. Later it was found that another Arf, Arf6, was localized to the PM and on endosomal membranes where it affected endosomal membrane traffic and the actin cytoskeleton [6].

Address: Building 50, Room 2503, Bethesda, MD 20892, USA, 301-402-2907 ph, 302-402-1519 fax, jdonalds@helix.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The importance of membrane lipids in facilitating membrane traffic events has long been acknowledged. The inter-conversion of phosphatidylinositol phosphates (PIPs) and the functions for distinct PIPs in establishing membrane identity has been examined at the Golgi complex where phosphatidylinositol 4-phosphate (PI4P) predominates and at the PM and endosomal systems where phosphatidylinositol 4,5-bisphosphate (PI4,5P₂) and phosphatidylinositol 3-phosphate (PI3P) reside, respectively [7]. These lipids are especially important for the membrane binding of some regulatory and trafficking machinery proteins, mediated through their pleckstrin homology (PH) domains [8]. On the other hand, PLD generates PA and DAG, conical-shaped lipids that favor negative curvature, and thus could affect membrane curvature, vesicle budding, fission and fusion [1,2,9].

This review will focus on studies that have demonstrated a role for PLD and its products PA and DAG in endocytosis and the trafficking of endosomal membrane. PLD is activated at the PM by signaling receptors, and hence, the generation of PA at the PM can serve as a second messenger during signal transduction. However PLD may also regulate membrane trafficking steps both constitutively and during signaling. There is also evidence for a role for PLD in other membrane trafficking activities in cells including phagocytosis, transport within the Golgi complex and regulated secretion. These topics will be discussed in other reviews in this issue.

2. Overview of Endocytic Pathways

Cells internalize PM proteins and lipids, ligands, and extracellular fluid through different endocytic mechanisms (Fig. 1). The itinerary of the cargo that enters cells through these mechanisms varies, but in general, membrane and content can be delivered to late endosomes for degradation, sent to the Golgi complex or recycled back out to the PM. These endosomal pathways serve to bring nutrients into cells, redistribute PM proteins to other cell surface domains, downregulate signaling receptors, and turnover membrane proteins and lipids [10, 11]. Through these pathways the composition of the cell surface can be modified for appropriate cell signaling or alterations in cell shape. There are many forms of endocytosis but they can be organized into the two categories of clathrin-dependent and clathrin-independent.

We know a great deal about clathrin-dependent endocytosis and the fate of proteins trafficking along these pathways. PM proteins that contain amino acid sequences in their cytoplasmic tail that bind to adaptor proteins (AP), such as AP2, can be recognized and sorted into clathrin coated regions of the PM and enter cells in clathrin-coated vesicles (Fig. 1). This process is selective and highly efficient and is facilitated by a number of other accessory proteins including the dynamin GTPase that is required for vesicle fission [10,11]. Examples of proteins entering cells by clathrin-dependent endocytosis include the transferrin and LDL receptors and many G protein-coupled receptors (GPCRs) after agonist stimulation. Immediately after endocytosis, the vesicles lose the clathrin coat and then fuse with the early endosome (EE), sometimes referred to as the sorting endosome. Rab5, PI3P and a number of other regulatory and coat proteins associate with the EE and facilitate the sorting of cargo proteins for their transport to the trans Golgi network, late endosomes, or juxtanuclear endocytic recycling compartment (ERC) for transport back to the PM.

Much less is known about clathrin-independent endocytosis pathways. These pathways include some specialized pathways such as caveolar endocytosis and the actin driven processes of macropinocytosis and phagocytosis. With the exception of caveolin-mediated endocytosis, most clathrin-independent endocytosis occurs independently of dynamin. Some clathrin-independent endocytic pathways have been characterized in studies following the endocytosis of lipid-binding toxins or transfected proteins in various cells types and the reader is referred to a few general reviews [12,13]. We and others have examined clathrin-independent

endocytosis using HeLa cells as a model system and have identified a number of endogenous PM proteins that enter cells by this mechanism (Figure 1), including the major histocompatibility class I proteins (MHCI) [14,15], the GPI-anchored protein CD59 [16], integrins [17], E-cadherin [18] and syndecan I [19]. Internalization is independent of dynamin and clathrin, but requires free PM cholesterol [14,16], a feature shared by internalization of so-called "raft-associated" proteins. After endocytosis, the vesicles carrying the cargo proteins fuse with the EE and from there the cargo is either routed to LE for degradation or recycled via the ERC back to the PM in distinctive tubular/vesicular carriers [20]. In HeLa cells Arf6 is associated with the clathrin-independent pathway and Arf6 activation is required for recycling back to the PM [15]. In addition to enabling endocytosed membrane to recycle, Arf6-GTP at the PM enhances many actin rearrangements including those associated with Rac1 such as PM ruffling, cell adhesion, wound healing, cell migration, invasion and metastasis [6,21].

3. PLD Structure, Localization & Functions

There are two PLD genes in humans, PLD1 and PLD2, and they are believed to be widely expressed in nearly all tissues [1,2]. In biochemical assays, PLD1 has low basal activity that can be stimulated by Arf and Rho GTPases and by protein kinase C (PKC). Although PLD2 has higher basal activity in biochemical assays than PLD1, studies with amino terminal truncations of PLD2 revealed that such forms can be activated by Arf proteins [22] and other studies have shown that PLD2 can be regulated in cells, in particular by Arf6 [23]. The two PLDs have similar structure with amino terminal phox consensus sequence (PX) and pleckstrin homology (PH) domains, which bind to phosphatidylinositol 3,4,5-trisphosphate and PI4,5P₂ respectively, followed by two catalytic domains in the second half of the protein. The amino terminal region is important for membrane localization and, in PLD1 is the site where protein kinase C (PKC) binds to activate PLD. The second catalytic half of the protein is the site where PI4,5P₂ and, in PLD1, Arf and Rho, bind to activate PLD.

Determining the cellular localization of endogenous PLD1 and PLD2 has been hampered by a lack of good immunological reagents and low abundance of these proteins. Many studies have utilized expression of epitope-tagged PLDs to determine likely cellular location, although there is some concern that overexpression might alter localization [9]. In general, PLD1 colocalizes with juxtanuclear structures that include late endosomes and the Golgi complex and can be observed recruited to the PM during stimulation [24–26]. PLD2 has been consistently localized to the PM and to endosomal structures [27,28]. Interestingly, expressed PLD2 has been shown to colocalize with Arf6 on tubular endosomal membranes in HeLa cells [23,29] suggesting that PLD2 is present at the PM and on the clathrin-independent endosomal pathway.

The PA and DAG that is generated as a result of PLD activity can affect membrane trafficking directly by altering membrane curvature or indirectly by recruiting and/or activating proteins (Fig. 2). PA and DAG both promote negative membrane curvature, the highly curved membrane neck that appears during vesicle fission and the intermediate stalk that appears during vesicle fusion [9]. Additionally a number of proteins bind to PA including Arf, the NEM-sensitive factor NSF involved in the vesicle fusion cycle [30], AP2 itself, the mammalian target of rapamycin (mTOR) [31], and the PH domain of SOS, the Ras guanine nucleotide exchange factor [32]. PA can activate PI4P5-kinase, the enzyme that produces PI4,5P₂ [33], and PA and DAG can stimulate the activity of some Arf GTPase activating proteins (GAPs) [34]. Since Arfs can activate both PLD [3,4] and PI4P5-kinase [35,36], the PA and PI4,5P₂ generated can feedback negatively on Arf through Arf GAPs and positively on the PI4P5-kinase and PLD.

Studies of PLD function in cells have relied on a number of molecular, biochemical and pharmacological approaches that together have been informative. Overexpression of epitope-tagged wild type or catalytically-inactive mutants have in some cases revealed the consequence of increased or decreased PLD activity respectively. The use of siRNA to knock-down levels of PLD1 and 2 has provided a new tool to probe cellular function. Both of the above approaches are complemented by pharmacological reagents that perturb PLD activity. During PLD stimulation of PC hydrolysis, primary alcohols can replace water in the transphosphatidyl transfer reaction forming phosphatidylbutanol instead of PA. This reaction serves as the basis for the biochemical assay for PLD activity. Cells can be treated with primary alcohols such as 1-butanol and this blocks the formation of PA and any subsequent conversion to DAG. By contrast, treatment of cells with secondary alcohols does not have this effect and can thus serve as controls. Additionally the conversion of PA to DAG is controlled by PA phosphohydrolase, which can be inhibited by propranolol and the DAG to PA reaction catalyzed by DAG kinase can be inhibited as well. Finally expression of mutant forms of the PLD regulators, Rho, Arf, Rac, and PKC, or pharmacologic treatments that alter the activity of these regulators, can also be used to understand how PLD is regulated and functions. Although each of these approaches has problems and caveats associated with them, used as an ensemble, they are effective tools for teasing out cellular functions for PLD.

4. PLD in internalization of signaling receptors

Interestingly, all of the studies that link PLD with the endocytosis step pertain to internalization of signaling receptors. These investigations may have been initiated due to the fact that activation of these receptors leads to activation of PLD. In 2001 Shen et al [37] reported that internalization and degradation of the EGF receptor (EGFR) was enhanced by overexpression of either PLD1 or PLD2, and inhibited by expression of catalytically inactive mutants of PLD or treatment of cells with primary alcohols. Additionally they showed that inhibition of Rac or PKC, activators of PLD, also inhibited endocytosis of EGFR. PLD2 has also been shown to be required for agonist stimulated endocytosis of several GPCRs. The μ -opioid receptor was shown to associate with PLD2, and receptor endocytosis was impaired in cells expressing mutants of PLD2 or treated with primary alcohols [38]. Depletion using siRNA of PLD2, but not of PLD1, blocked agonist-induced endocytosis of the angiotensin II receptor [28]. In each of these cases, the form of endocytosis is likely to be clathrin-dependent and this suggests that during agonist-induced receptor endocytosis PLD2 is required. On the other hand, depletion of PLD1 in B cells inhibits endocytosis of the B cell receptor, although the endocytic mechanism involved in internalization of this receptor is not entirely clear [39].

Interestingly, Bhattacharya et al [40] reported that PLD2 colocalized with the metabotropic glutamate receptors 1 and 5 and was required for the constitutive internalization of these receptors, which they demonstrated by siRNA depletion of PLD2 and by alcohol treatment. The constitutive endocytosis of the μ -opioid receptor was also shown to be dependent on PLD2 [41]. It is not entirely clear if this constitutive endocytosis of these receptors is still through clathrin-dependent mechanisms. However, it was recently reported that transfected β_2 -adrenergic receptor and the M3 muscarinic receptor traffic constitutively through the clathrin-independent endocytosis in HeLa cells [42] where they colocalize with PLD2 [29].

A new twist on this story of receptor endocytosis requiring PLD2 was provided by Lee et al [43]. They demonstrated that PLD1 and 2 bind to dynamin in its GTP-bound form and stimulate its GTPase activity and that it is the PX domain in PLD that is responsible for this activity. Expression of either PX domain alone led to a stimulation in EGFR endocytosis, similar to the effect previously observed upon overexpression of PLD1 or 2 by Shen et al [37]. Furthermore, knockdown of both PLD1 and 2 led to a decrease in EGFR endocytosis that could be rescued by expression of siRNA-resistant constructs of wild type PLD or lipase inactive PLD [43].

This suggests that PLD could influence clathrin-dependent endocytosis or other dynamin-dependent forms of endocytosis independently of its ability to generate PA or DAG. In light of this observation, some of the results above might need to be re-examined. It is possible, however, that PLD may be having multiple effects on endocytosis with PA important for generation of negative membrane curvature at the vesicle neck and PLD itself engaging dynamin to facilitate vesicle scission (Fig. 1).

5. PLD in endocytic membrane recycling

In addition to endocytosis, there is evidence that PLD, and the PA and DAG produced, are required for endosomal recycling pathways. Two studies examined recycling of PM proteins that constitutively recycle in HeLa cells, one in clathrin-dependent and the other in the clathrin-independent pathway.

Recycling of the transferrin receptor after endocytosis is very efficient and occurs from two sites: a rapid recycling from the EE that involves Rab4 and a slower recycling from the ERC involving Rab11. Using siRNA, depletion of PLD2 from HeLa cells inhibited recycling of the transferrin receptor from the ERC but did not appear to affect endocytosis [44]. Knock-down of PLD1 had no effect on transferrin trafficking. The authors also found that the depletion of PLD2, but not PLD1, decreased PA levels in the non-stimulated cells suggesting that it is PLD2 that contributes to PA levels in resting cells. The PI4,5P₂ levels were also lower in PLD2 depleted cells indicating that PA could be a significant stimulator of PI4P5kinase in cells [44]. This study shows that PLD2 was important for the recycling of transferrin, but it did not address whether the PLD2 and PA produced were required on the endosome or at the PM.

Another study examined PLD functioning as a consequence of Arf6 activities in the clathrin-independent endocytic recycling pathway (Fig. 1). As mentioned previously, activation of Arf6 is required for recycling of membrane proteins back to the PM [15,17,45,46]. In HeLa cells inhibition of recycling causes a build up of membranous tubules in the cells that carry recycling cargo such as MHCI. Treatment of cells with 1-butanol also caused a build-up of tubules and a measured block in recycling of MHCI [47]. Additionally, expression in cells of an effector domain mutant of Arf6, N48R, which cannot activate PLD [36,48], recreated this block in transport (see Fig. 1 and Fig 2). This mutant of Arf6 can still be activated by Arf6 guanine nucleotide exchange factors and inhibited by GTPase activating proteins [49] and can still activate PI4P5-kinase [36,48] so it is a valuable mutant for teasing out specific Arf functions. The block in membrane recycling induced by expression of Arf6N48R could be relieved if cells were treated with the phorbol ester PMA, which leads to the activation of PLD through activation of PKC, thus by-passing the need for Arf6 stimulation of PLD [47]. Interestingly, the use of propranolol to block the conversion of PA to DAG (Fig. 2), did result in the break up of the tubular recycling endosomes into vesicles (depicted in Fig. 1 as vesicles close to the PM), but did not relieve the block in fusion of the vesicles containing MHCI with the PM, suggesting that PA-derived DAG might be required for vesicle fusion. Although PLD was not knocked down in this study as it was in the previously mentioned study [44], it seems likely that it is PLD2 and not PLD1 that is being affected by Arf6 since PLD2 is associated with these endosomal membranes. These clathrin-independent endosomal membranes also have associated with them PI4P5-kinase [45], H-Ras [29], Src [45] and other signaling components that could impact PLD activities [50]. The requirements for Arf6 and PLD for recycling of clathrin-independent derived endosomes is reminiscent of the requirements for regulated secretion in neuroendocrine cells and a recent study using siRNA of Arf6 confirms that Arf6 facilitates exocytosis through activation of PLD1 [51]

The colocalization of Arf6 and PLD2 at the PM and on these endosomal membranes suggests that Arf6 is influencing PLD2 localization. Furthermore, the recycling of clathrin-independent

endosomes back to the PM is important to deliver membrane containing PLD2, PI4P5-kinase, Ras and integrins back to the PM. These components can then be important for the changes in the actin cytoskeleton that can be initiated by activation of Arf6, Rac, or Ras. Arf6-stimulated cell migration in MDCK cells occurs through activation of both Rac and PLD [52] and antigen-stimulated Mast cells exhibit PM ruffles that is dependent upon Arf6 and PLD2 [27]. Although these changes in cortical actin structure are occurring at the PM, the recycling of endosomal membrane is probably important for these processes.

6. Perspectives for the future

It is clear that the activities of PLD and its product PA affect membrane traffic at the PM. The localization of PLD2, and after stimulation PLD1, to the PM places these enzymes in a site where their activities could affect both endocytosis from the PM and the fusion of recycled endosomes back to the PM. The observation that in HeLa cells expressed PLD2 can also enter cells and traffic along the clathrin-independent endocytic pathway raises the possibility that PLD can function on these endosomes, but more studies and in other cell types are needed. Additional studies taking multi-faceted approaches of knock-down, expression of mutants and pharmacologic approaches need to be performed to be sure that the phenotypes attributed to PLD or Arf6 are due to direct effects and not indirect effects. This may be especially important with regards to the inter-dependence of clathrin-dependent and clathrin-independent membrane systems. Finally, understanding where PA and DAG are formed and how they facilitate vesicle fission and fusion will be facilitated by the use of fluorescent probes for detecting these bioactive lipids in living cells.

References

1. Jenkins GM, Frohman MA. Phospholipase D: a lipid centric review. *Cell Mol Life Sci* 2005;62:2305–2316. [PubMed: 16143829]
2. McDermott M, Wakelam MJ, Morris AJ. Phospholipase D. *Biochem Cell Biol* 2004;82:225–253. [PubMed: 15052340]
3. Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 1993;75:1137–1144. [PubMed: 8261513]
4. Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E, Gout I, Hiles I, Totty NF, Truong O, Hsuan JJ. Phospholipase D: a downstream effector of ARF in granulocytes. *Science* 1994;263:523–526. [PubMed: 8290961]
5. Donaldson JG, Klausner RD. ARF: a key regulatory switch in membrane traffic and organelle structure. *Curr Opin Cell Biol* 1994;6:527–532. [PubMed: 7986529]
6. Donaldson JG. Multiple roles for Arf6: Sorting, structuring, and signaling at the plasma membrane. *Journal of Biological Chemistry* 2003;278:41573–41576. [PubMed: 12912991]
7. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature* 2006;443:651–657. [PubMed: 17035995]
8. Balla T. Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J Cell Sci* 2005;118:2093–2104. [PubMed: 15890985]
9. Roth MG. Molecular mechanisms of PLD function in membrane traffic. *Traffic* 2008;9:1233–1239. [PubMed: 18422860]
10. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003;422:37–44. [PubMed: 12621426]
11. Slepnev VI, De Camilli P. Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nature Reviews Neuroscience* 2000;1:161–172.
12. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* 2007;8:603–612. [PubMed: 17609668]

13. Sandvig K, Torgersen ML, Raa HA, van Deurs B. Clathrin-independent endocytosis: from nonexistent to an extreme degree of complexity. *Histochem Cell Biol* 2008;129:267–276. [PubMed: 18193449]
14. Naslavsky N, Weigert R, Donaldson JG. Convergence of Non-clathrin- and Clathrin-derived Endosomes Involves Arf6 Inactivation and Changes in Phosphoinositides. *Mol Biol Cell* 2003;14:417–431. [PubMed: 12589044]
15. Radhakrishna H, Donaldson JG. ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J Cell Biol* 1997;139:49–61. [PubMed: 9314528]
16. Naslavsky N, Weigert R, Donaldson JG. Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol Biol Cell* 2004;15:3542–3552. [PubMed: 15146059]
17. Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, Hsu VW. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic* 2004;5:20–36. [PubMed: 14675422]
18. Paterson AD, Parton RG, Ferguson C, Stow JL, Yap AS. Characterization of E-cadherin Endocytosis in Isolated MCF-7 and Chinese Hamster Ovary Cells: THE INITIAL FATE OF UNBOUND E-CADHERIN. *J Biol Chem* 2003;278:21050–21057. [PubMed: 12657640]
19. Zimmermann P, Zhang Z, Degeest G, Mortier E, Leenaerts I, Coomans C, Schulz J, N'Kuli F, Courtoy PJ, David G. Syndecan recycling [corrected] is controlled by syntenin-PIP2 interaction and Arf6. *Dev Cell* 2005;9:377–388. [PubMed: 16139226]
20. Weigert R, Yeung AC, Li J, Donaldson JG. Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol Biol Cell* 2004;15:3758–3770. [PubMed: 15181155]
21. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol* 2006;7:347–358. [PubMed: 16633337]
22. Sung TC, Altshuler YM, Morris AJ, Frohman MA. Molecular analysis of mammalian phospholipase D2. *J Biol Chem* 1999;274:494–502. [PubMed: 9867870]
23. Hiroyama M, Exton JH. Localization and regulation of phospholipase D2 by ARF6. *J Cell Biochem* 2005;95:149–164. [PubMed: 15759270]
24. Brown FD, Thompson N, Saqib KM, Clark JM, Powner D, Thompson NT, Solari R, Wakelam MJ. Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. *Curr Biol* 1998;8:835–838. [PubMed: 9663393]
25. Du G, Altshuler YM, Vitale N, Huang P, Chasserot-Golaz S, Morris AJ, Bader MF, Frohman MA. Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. *J Cell Biol* 2003;162:305–315. [PubMed: 12876278]
26. Freyberg Z, Sweeney D, Siddhanta A, Bourgoin S, Frohman M, Shields D. Intracellular localization of phospholipase D1 in mammalian cells. *Mol Biol Cell* 2001;12:943–955. [PubMed: 11294898]
27. O'Lunaigh N, Pardo R, Fensome A, Allen-Baume V, Jones D, Holt MR, Cockcroft S. Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol Biol Cell* 2002;13:3730–3746. [PubMed: 12388770]
28. Du G, Huang P, Liang BT, Frohman MA. Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. *Mol Biol Cell* 2004;15:1024–1030. [PubMed: 14718562]
29. Porat-Shliom N, Kloog Y, Donaldson JG. A Unique Platform for H-Ras Signaling Involving Clathrin-independent Endocytosis. *Mol Biol Cell* 2008;19:765–775. [PubMed: 18094044]
30. Manifava M, Thuring JW, Lim ZY, Packman L, Holmes AB, Ktistakis NT. Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)- bisphosphate-coupled affinity reagents. *J Biol Chem* 2001;276:8987–8994. [PubMed: 11124268]
31. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* 2001;294:1942–1945. [PubMed: 11729323]
32. Zhao C, Du G, Skowronek K, Frohman MA, Bar-Sagi D. Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat Cell Biol* 2007;9:706–712. [PubMed: 17486115]
33. Jenkins GH, Fiset PL, Anderson RA. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem* 1994;269:11547–11554. [PubMed: 8157686]

34. Inoue H, Randazzo PA. Arf GAPs and their interacting proteins. *Traffic* 2007;8:1465–1475. [PubMed: 17666108]
35. Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman MA, Kanaho Y. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* 1999;99:521–532. [PubMed: 10589680]
36. Skippen A, Jones DH, Morgan CP, Li M, Cockcroft S. Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J Biol Chem* 2002;277:5823–5831. [PubMed: 11744730]
37. Shen Y, Xu L, Foster DA. Role for phospholipase D in receptor-mediated endocytosis. *Mol Cell Biol* 2001;21:595–602. [PubMed: 11134345]
38. Koch T, Brandenburg LO, Liang Y, Schulz S, Beyer A, Schroder H, Holtt V. Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. *J Neurochem* 2004;88:680–688. [PubMed: 14720217]
39. Snyder MD, Pierce SK. A mutation in Epstein-Barr virus LMP2A reveals a role for phospholipase D in B-Cell antigen receptor trafficking. *Traffic* 2006;7:993–1006. [PubMed: 16882041]
40. Bhattacharya M, Babwah AV, Godin C, Anborgh PH, Dale LB, Poulter MO, Ferguson SS. Ral and phospholipase D2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis. *J Neurosci* 2004;24:8752–8761. [PubMed: 15470141]
41. Koch T, Wu DF, Yang LQ, Brandenburg LO, Holtt V. Role of phospholipase D2 in the agonist-induced and constitutive endocytosis of G-protein coupled receptors. *J Neurochem* 2006;97:365–372. [PubMed: 16539674]
42. Scarselli M, Donaldson JG. Constitutive internalization of G protein-coupled receptors and G proteins via clathrin-independent endocytosis. *J Biol Chem* 2009;284:3577–3585. [PubMed: 19033440]
43. Lee CS, Kim IS, Park JB, Lee MN, Lee HY, Suh PG, Ryu SH. The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis. *Nat Cell Biol* 2006;8:477–484. [PubMed: 16622417]
44. Padron D, Tall RD, Roth MG. Phospholipase D2 is required for efficient endocytic recycling of transferrin receptors. *Mol Biol Cell* 2006;17:598–606. [PubMed: 16291863]
45. Brown FD, Rozelle AL, Yin HL, Balla T, Donaldson JG. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol* 2001;154:1007–1017. [PubMed: 11535619]
46. Robertson SE, Setty SR, Sitaram A, Marks MS, Lewis RE, Chou MM. Extracellular signal-regulated kinase regulates clathrin-independent endosomal trafficking. *Mol Biol Cell* 2006;17:645–657. [PubMed: 16314390]
47. Jovanovic OA, Brown FD, Donaldson JG. An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. *Mol Biol Cell* 2006;17:327–335. [PubMed: 16280360]
48. Jones DH, Bax B, Fensome A, Cockcroft S. ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatamer I. *Biochem J* 1999;341(Pt 1):185–192. [PubMed: 10377261]
49. Vitale N, Chasserot-Golaz S, Bailly Y, Morinaga N, Frohman MA, Bader MF. Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. *J Cell Biol* 2002;159:79–89. [PubMed: 12379803]
50. Donaldson JG, Porat-Shliom N, Cohen LA. Clathrin-independent endocytosis: a unique platform for cell signaling and PM remodeling. *Cell Signal* 2009;21:1–6. [PubMed: 18647649]
51. Begle A, Tryoen-Toth P, de Barry J, Bader MF, Vitale N. ARF6 regulates the synthesis of fusogenic lipids for calcium-regulated exocytosis in neuroendocrine cells. *J Biol Chem*. 2009
52. Santy LC, Casanova JE. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol* 2001;154:599–610. [PubMed: 11481345]

Figure 1.

Model of PLD and clathrin-dependent and clathrin-independent endocytosis membrane systems. Clathrin-dependent endocytosis internalizes cargo such as the transferrin receptor (blue bars) and is dependent upon dynamin (black crescent). These endosomes subsequently fuse with the early endosome (EE) and transferrin receptor can be recycled back to the PM via the juxtanuclear endocytic recycling compartment (ERC). Clathrin-independent endocytosis internalizes cargo such as MHC I (red bars) into cells independently of dynamin. These endosomes fuse with EE and MHC I can also recycle back to the PM but in distinct tubular membrane carriers devoid of transferrin receptor. PLD1 (blue boxes) is shown associated with late endosomes and also at the PM. PLD2 (green boxes) is shown at the PM and also associated with clathrin-independent endosomes observed in HeLa cells. Possible sites of PA and DAG generation (yellow stars).

Figure 2.

Arf6 and PLD network of signaling. The Arf6-GDP/GTP cycle and inter-conversion of PA and DAG are shown. Other arrows indicate stimulation of activity. PA generated from PLD stimulates Arf GAP and PI4P5-kinase. Inhibitors indicated with a "T". DGK, DAG kinase; PAP, PA phosphohydrolase.