Class III PI-3-kinase activates phospholipase D in an amino acid–sensing mTORC1 pathway

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The rapamycin-sensitive mammalian target of rapamycin (mTOR) complex, mTORC1, regulates cell growth in response to mitogenic signals and amino acid availability. Phospholipase D (PLD) and its product, phosphatidic acid, have been established as mediators of mitogenic activation of mTORC1. In this study, we identify a novel role for PLD1 in an amino acid–sensing pathway. We find that amino acids activate PLD1 and that PLD1 is indispensable for amino acid activation of mTORC1. Activation of PLD1 by amino acids requires the class III phosphatidylinositol 3-kinase hVps34, which stimulates PLD1 activity through a functional interaction between phosphatidylinositol 3-phosphate and the Phox homology (PX) domain of PLD1. Furthermore, amino acids stimulate PLD1 translocation to the lysosomal region where mTORC1 activation occurs in an hVps34-dependent manner, and this translocation is necessary for mTORC1 activation. The PX domain is required for PLD1 translocation, mTORC1 activation, and cell size regulation. Finally, we show that the hVps34-PLD1 pathway acts independently of, and in parallel to, the Rag pathway in regulating amino acid activation of mTORC1.

Introduction

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase critically involved in the regulation of many cellular and developmental processes including cell growth, differentiation, and metabolism. Two functionally distinct protein complexes containing mTOR have been characterized, namely mTORC1 and mTORC2, which mediate the rapamycin-sensitive and -insensitive signaling of mTOR, respectively (Sarbassov et al., 2005a). mTORC1 assembles a signaling network in the regulation of cell growth by mediating nutrient availability (amino acid sufficiency) and mitogenic signals. The two best-characterized immediate targets of mTORC1 are ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor–4E-binding protein 1 (4E-BP1), both of which regulate protein synthesis at the translation initiation level (Hay and Sonenberg, 2004). The tumor suppressor tuberous sclerosis complex TSC1–TSC2 and the target of its GTPase-activating protein activity, Rheb, form a major hub that receives multiple upstream signals to activate mTORC1 (Manning and Cantley, 2003).

The sensing and transduction of amino acid signals upstream of mTORC1 have been an issue of long-standing interest, as this mechanistically less well-understood aspect of mTOR regulation represents a fundamentally important signaling process and may be intimately linked to human diseases such as cancer and metabolic syndromes. To date, two major pathways have been reported to mediate amino acid signals to activate mTORC1, involving the class III phosphatidylinositol 3-kinase (PI-3-kinase) human vacuolar protein sorting 34 (hVps34) and the Rag family of small G proteins. hVps34 has been found to be activated by amino acids and required for mTORC1 activation in response to amino acid stimulation (Byfield et al., 2005; Nobukuni et al., 2005). In vivo validation of hVps34 as a key regulator of mTORC1 came from a recent study showing that hVps34-deficient embryos had drastically reduced levels of S6 phosphorylation and were defective in cell proliferation (Zhou et al., 2011). As upstream regulators, calcium and CaM have been shown to bind and activate hVps34 (Gulati et al., 2008).
but others have questioned this mode of hVps34 regulation (Yan et al., 2009). Curiously, Vps34 does not regulate TOR signaling in Drosophila melanogaster (Juhasz et al., 2008), suggesting that the hVps34-mTOR regulatory branch may have evolved to accommodate the biological complexity in higher organisms. The Rag GTPase heterodimers, through the P18–P14–MP1 complex, recruit mTORC1 to the lysosomal surface upon amino acid stimulation, where Rheb presumably resides and mTORC1 activation occurs (Kim et al., 2008; Sancak et al., 2008, 2010). The Ste20 kinase MAP4K3 and its inhibitor PP2A/PR61-ε have also been reported to mediate amino acid signaling to mTORC1 in a Rag-dependent manner, although they may constitute a pathway parallel to Rag (Findlay et al., 2007; Yan et al., 2010). It is not known how the hVps34 and Rag pathways are connected or how hVps34 activates mTORC1.

Mitogenic activation of mTORC1 also requires the lipid second messenger phosphatidic acid (PA), which binds to the FKBP12-rapamycin–binding domain of mTOR (Fang et al., 2001; Foster, 2007; Sun and Chen, 2008). Phospholipase D (PLD), catalyzing the hydrolysis of phosphatidylcholine to PA, has been established as a key upstream component in the mitogenic mTORC1 pathway that regulates cell growth (Fang et al., 2003; Sun and Chen, 2008). Like hVps34, PLD does not regulate TOR in Drosophila (Sun and Chen, 2008). Of the two mammalian isoforms of PLD, PLD2 displays a high basal activity in most mammalian cells, whereas PLD1 has little activity in resting cells and is activated by a variety of mitogens and agonists (Frohman et al., 1999). PLD1 has been found to be a Rheb effector, which directly connects the PA and tuberous sclerosis complex–Rheb pathways upstream of mTORC1 (Sun et al., 2008). Here, we report a novel role of PLD1 in transducing amino acid signals to activate mTORC1 via an hVps34–phosphatidylinositol 3-phosphate (PI3P)–PLD1 pathway.

**Results**

**PLD1 lies in an amino acid-sensing mTORC1 pathway**

The PLD/PA axis has long been considered a mitogenic pathway that acted in parallel to amino acid–sensing pathways upstream of mTORC1 (Fang et al., 2001, 2003); PLD had not been directly connected to amino acid signals. However, our recent observation that serum stimulation of PLD activation was dependent on the presence of amino acids (Sun et al., 2008) prompted us to reexamine the relationship between PLD and amino acids. As shown in Figure 1 A, amino acid withdrawal diminished basal cellular PLD activity in serum-starved HEK293 cells. Furthermore, in serum-starved and amino acid–deprived cells, amino acids at the concentrations found in DME acutely stimulated PLD activity by 1.5-fold (Fig. 1 A), and this activation was also observed to various degrees in four other mouse and human cell lines (C2C12, HepG2, HeLa, and 3T3L1; Fig. 1 B). It is important to note that this seemingly modest degree of activation by amino acids is comparable with those induced by other well-known PLD stimuli such as insulin (Fig. 1 A; Voss et al., 1999) and serum (Kötter et al., 2000; Sun et al., 2008). To distinguish between the two mammalian isoforms of PLD, we measured in vivo activities of recombinant proteins. As shown in Figure 1 C, PLD1 was stimulated by amino acids, whereas PLD2 displayed a higher unstimulated activity that was not affected significantly by amino acids (Fig. 1 C). Thus, PLD1 appears to be the amino acid–sensitive isoenzyme.

To examine a potential role of PLD1 in the amino acid–sensing mTORC1 pathway, we knocked down PLD1 by lentivirus-mediated expression of small hairpin RNA (shRNA) and found amino acid–induced S6K1 phosphorylation at Thr389 and 4E-BP1 phosphorylation at Thr37/46, both common readouts for mTORC1 activity, to be significantly decreased (Fig. 1 D). The effect was observed with two independent shRNAs, validating the specificity of the RNAi. Leu-stimulated phosphorylation of S6K1 and 4E-BP1 was also inhibited by PLD1 knockdown (Fig. 1 E). Knockdown of PLD1 blocked amino acid activation of S6K1 in four other cell lines as well (Fig. 1 F). Knockdown of PLD2, on the other hand, did not have any effect on amino acid stimulation (Fig. S1), which is consistent with the lack of PLD2 activation by amino acids (Fig. 1 C). It should be noted that endogenous PLD2 activity in HEK293 cells is very low, and the protein is difficult to detect (Slabaey et al., 2000), so that PLD1 is likely the major contributor to the total cellular PLD activity in these cells. So far, our results placed PLD1 downstream of amino acid signals that activate mTORC1.

**hVps34 is necessary for amino acid activation of PLD1 upstream of mTORC1**

In searching for a mechanism by which PLD1 might be activated by amino acid signals upstream of mTORC1, we considered one of the reported regulators in this pathway, hVps34 (Byfield et al., 2005; Nobukuni et al., 2005). Amino acid activation of hVps34 leads to increased production of PI3P, and PI3P is known to regulate its effector proteins by binding to their Fab1/YOTB/Vac1/EEA1 (FYVE) or Phox homology (PX) domains (Lemmon, 2003; Backer, 2008). As PLD1 contains a PX domain, we wondered whether hVps34 could be linked to PLD1 through a PI3P–PX interaction. To probe this possibility, we first examined whether hVps34 lied upstream of PLD1 in the mTORC1 pathway. 3-methyladenine (3-MA), an hVps34 inhibi- tor (Miller et al., 2010), blocked amino acid–stimulated cellular PLD activity, whereas rapamycin had no effect (Fig. 2 A). Wortmannin, another PI3-kinase inhibitor, had a similar effect on PLD as 3-MA (unpublished data). At the same time, S6K1 phosphorylation was abolished by 3-MA as well as by rapamycin, as expected. As neither 3-MA nor any other known inhibitor is absolutely specific for hVps34, we sought definitive evidence for hVps34 involvement by knockdown, which indeed inhibited amino acid activation of cellular PLD (Fig. 2 B). Insulin stimulation of cellular PLD was also blocked by hVps34 knockdown (Fig. 2 C), consistent with the notion that mitogenic stimulation of PLD is dependent on amino acid sufficiency. hVps34 knockdown inhibited amino acid– and insulin-stimulated S6K1 phosphorylation (Fig. 2, B and C) but not insulin-stimulated Akt phosphorylation (Fig. 2 C), confirming the previously reported critical role of hVps34 in mTORC1 signaling (Byfield et al., 2005; Nobukuni et al., 2005). Collectively, our results strongly suggest that PLD lies downstream of...
It is noteworthy that exogenous PA could activate mTORC1 signaling in the absence of mitogens (Fig. 2 E; Fang et al., 2001) but not in the absence of amino acids (Fig. 2 F, first through fourth lanes). In other words, PA is sufficient to act as a mitogen in the mTORC1 pathway, but it cannot replace amino acid signals, suggesting that other amino acid–sensing pathways—for instance, the Rag pathway (Sancak et al., 2008, 2010)—may be required in parallel with the PLD-PA pathway to transduce amino acid signals to mTORC1.

Next, we asked whether PLD played a role in connecting hVps34 to mTORC1 in the amino acid–sensing pathway. Overexpression of recombinant hVps34 enhanced the amino acid–induced activation of S6K1 (Fig. 2 D), as previously reported (Nobukuni et al., 2005). In these experiments, we overexpressed hVps34 and hVps15 bicistronically because coexpression of hVps15 resulted in higher specific activity of the recombinant hVps34 (Yan et al., 2009). The hVps34–induced S6K1 phosphorylation was abolished by PLD1 knockdown (Fig. 2 D), suggesting that PLD1 is necessary for hVps34 activation of mTORC1. Although hVps34 knockdown suppressed insulin- and amino acid–induced S6K1 phosphorylation (Fig. 2, B and C), it did not affect the activation of S6K1 by exogenous PA (Fig. 2 E), which is consistent with PLD lying downstream of hVps34 and upstream of mTORC1. Furthermore, exogenous PA rescued amino acid–induced S6K1 phosphorylation from hVps34 knockdown (Fig. 2 F, last four lanes). Collectively, these observations put PLD1 between hVps34 and mTORC1 in a pathway regulated by amino acids.

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**hVps34 activates PLD1 through PI3P**

Further probing the relationship between hVps34 and PLD1, we overexpressed hVps34/hVps15 and examined the effect on PLD1 activity. hVps34 overexpression in HEK293 cells stimulated PLD1 activity in a dose-dependent manner accompanied by increased S6K1 phosphorylation, as expected (Fig. 3 A). hVps34 overexpression also augmented amino acid–induced PLD1 activity (Fig. 3 B). These results are consistent with hVps34 being an activator of PLD1 in an amino acid–sensing pathway. To assess whether the kinase activity of hVps34 was required for PLD1 activation, we knocked down endogenous
hVps34 and then expressed RNAbi-resistant recombinant hVps34, either wild-type (wt) or kinase-dead. hVps34 knockdown impaired amino acid–induced PLD activation, as shown earlier (Fig. 2 B), and the expression of recombinant wt-hVps34 rescued PLD activation (Fig. 3 C), further validating target specificity of the hVps34 knockdown. Importantly, expression of the kinase-dead hVps34 did not rescue PLD activity from the knockdown (Fig. 3 C), suggesting that activation of PLD1 requires the kinase activity of hVps34.

The requirement of hVps34 kinase activity implies that its product, PI3P, may regulate PLD. FYVE domains, when overexpressed, could sequester cellular PI3P and dominantly interfere with functions of endogenous PI3P-targeting proteins. We found that overexpression of an EGFP-2xFYVE fusion protein in HEK293 cells dampened S6K1 phosphorylation stimulated by amino acids (Fig. 4 A), which is consistent with previous studies (Byfield et al., 2005; Nobukuni et al., 2005). Importantly, amino acid activation of PLD, as well as hVps34–augmented PLD activity, was significantly suppressed by FYVE overexpression (Fig. 4 B). These observations are consistent with PI3P being a key regulator of PLD activity. To gain more direct insight into the role of PI3P in regulating PLD1, we delivered PI3P into HEK293 cells using a polyamine carrier (Ozaki et al., 2000). As shown in Fig. 4 C, exogenous PI3P activated cellular PLD1 activity by approximately twofold in the absence of amino acids as well as under serum starvation conditions, whereas carrier alone or with PI had no effect. Furthermore, exogenous PI3P stimulated PLD1 activity equally well in hVps34 knockdown cells (Fig. 4 D), suggesting that PI3P was sufficient for PLD1 activation in the absence of hVps34. Collectively, our results strongly suggest that hVps34 activates PLD1 through its product PI3P.
The PX domain of PLD1 is required for its activation by hVps34

The involvement of PI3P in the activation of PLD1 prompted us to ask whether this regulation is through the PX domain of PLD1. We examined three PLD1 mutants: PX deleted (ΔPX), R118G, and F120A/R179Q (Fig. 5 A). The latter two contained point mutations in the PX domain at the residues predicted to be required for interaction with phosphoinositide lipid anchors based on the published structures of other PX domains and sequence alignment of known PX domains (Sato et al., 2001; Du et al., 2003). The ΔPX mutant displayed higher basal activity compared with wt PLD1, as previously reported (Sung et al., 1999), and so did the two point mutants (compare the gray bars in Fig. 5 B). Interestingly, predicted molecular masses of the proteins are indicated for Western blots. S6K1 migrated on SDS-PAGE as a 70-kD protein.

Figure 3. hVps34 activates PLD1 in cells. (A) HEK293 cells were cotransfected with HA-PLD1 and increasing amounts of bicistronic Myc-hVps34/ V5-hVps15 cDNA followed by in vivo PLD assays and Western analysis of cell lysates in parallel samples. (B) Cells were cotransfected with HA-PLD1 and Myc-hVps34/V5-hVps15, serum starved overnight, amino acid (AA) deprived for 2 h, and then stimulated with amino acids for 30 min. Western analysis was performed. (C) Cells were transduced with lentiviruses expressing hVps34-shRNA, selected with puromycin, and transfected with wt or kinase-dead Myc-hVps34 followed by serum starvation overnight, amino acid deprivation for 2 h, and then stimulation with amino acids for 30 min. In vivo PLD assays and Western analysis of cell lysates were performed in parallel. Scram, scrambled. (A–C) All data are mean ± SD or representative blots from three to five independent experiments. A one-sample or paired t test was performed to compare the indicated pairs of data. *, P < 0.05; **, P < 0.01. Predicted molecular masses of the proteins are indicated for Western blots. S6K1 migrated on SDS-PAGE as a 70-kD protein.

Figure 4. PI3P activates PLD1 in cells. (A) HEK293 cells were cotransfected with HA-S6K1 and GFP-2xFYVE (the latter at two different amounts), serum starved overnight, amino acid (AA) deprived for 2 h, and then stimulated with amino acids for 30 min. Western analysis was performed. (B) Cells were transfected with GFP-FYVE at two different amounts with or without Myc-hVps34/V5-hVps15 cDNA and then treated as in A. In vivo PLD assays were performed. (C) Cells were transfected with HA-PLD1 followed by serum starvation overnight or amino acid withdrawal for 2 h. The cells were then stimulated with 15 µM PI3P or PI or carrier alone for 30 min. In vivo PLD assays were performed. (D) Cells were transduced with lentiviruses expressing hVps34-shRNA or scramble (scram), selected with puromycin, and then transfected with HA-PLD1 followed by serum starvation overnight and amino acid withdrawal for 2 h. The cells were then stimulated with 15 µM PI3P or carrier alone in the presence of amino acids for 30 min. In vivo PLD assays were performed. (B–D) All data are mean ± SD or representative blots from three to five independent experiments. A one-sample or paired t test was performed to compare the indicated pairs of data. *, P < 0.05; **, P < 0.01. Predicted molecular masses of the proteins are indicated for Western blots. S6K1 migrated on SDS-PAGE as a 70-kD protein.
We also found that hVps34 and PLD1 coimmunoprecipitated. Recombinant Myc-hVps34 specifically pulled down endogenous PLD1, and, conversely, recombinant Flag-PLD1 pulled down endogenous hVps34 (Fig. 5 D). The interaction between PLD1 and hVps34 was not regulated by amino acid signals, nor was it dependent on the PX domain of PLD1 (unpublished data). Although the functional significance of this interaction remains uncertain, it is possible that the constitutive association of hVps34 and PLD1 offers an advantage of proximity in the regulation of PLD1 by hVps34-produced PI3P.

Amino acids regulate PLD1 translocation to the lysosomal area in an hVps34-dependent fashion

It had been reported that amino acid signals, through the small GTPases Rag and the P18–P14–MP1 (Ragulator) complex, induce translocation of raptor/mTOR to the late endosomal/lysosomal region where activation of mTORC1 occurs (Sancak et al., 2008, 2010). We wondered whether PLD1, as a key regulator of mTORC1 activation, also underwent subcellular
translocation. PLD1 had been reported to reside on endomembranes including Golgi, ER, and late endosomes (Colley et al., 1997; Brown et al., 1998; Toda et al., 1999; Freyberg et al., 2001). Because an antibody that specifically recognizes endogenous PLD1 by immunostaining was not available, we expressed recombinant HA-PLD1 and examined its localization by anti-HA staining. When cells were deprived of both serum and amino acids, recombinant PLD1 was found in cytoplasmic puncta concentrated in the perinuclear region (Fig. 6 A), whereas mTOR was in puncta distributed throughout the cytoplasm (Fig. 6 B). Upon acute (30 min) stimulation by amino acids, some of the mTOR protein translocated to the lysosomal region marked by LAMP2 (Fig. 6 B), which is consistent with previous studies (Sancak et al., 2008, 2010; Flinn et al., 2010). Importantly, translocation of PLD1 to the LAMP2-positive lysosomal region upon amino acid stimulation was also clearly evident (Fig. 6 A). Quantification of the imaging results showed that 76 and 78% of PLD1- and mTOR-staining cells, respectively, displayed this localization pattern upon amino acid stimulation (Fig. 6 D). As expected from their translocation into the LAMP2-positive region, the ΔPX mutant was dispersed throughout the cytoplasm in amino acid–deprived cells, and no change in this localization was detected upon amino acid stimulation (Fig. 7, A and B). The R118G mutant displayed localization identical to that of the ΔPX mutant both in the absence and presence of amino acids (Fig. 7, A and B). The Δloop mutant, on the other hand, translocated to the LAMP2-positive region upon amino acid stimulation (Fig. 7, A and B). Therefore, amino acid–induced PLD1 translocation to the lysosomal region is dependent on its PX domain as well as on hVps34 kinase, which is similar to the requirements for the catalytic activation of PLD1 by amino acids. Thus, we wondered whether catalytic activation of PLD1 might be a prerequisite for its translocation. As shown in Fig. S3, the catalytically inactive K898R PLD1 mutant (Sung et al., 1997) translocated to the LAMP2-positive region in response to amino acid stimulation, suggesting that the catalytic activity of PLD1 is not necessary for its subcellular translocation in response to amino acids.

Collectively, our data clearly indicate Vps34-mediated amino acid stimulation of PLD1 translocation to the lysosomal area. An important question was whether this subcellular translocation was functionally relevant to mTORC1 activation. To address this issue, it was necessary to separate the activity of PLD1 from its translocation, and the ΔPX mutant of PLD1 served that purpose. The activity of ΔPX-PLD1 was comparable with wt PLD1 upon amino acid stimulation, even though the mutant was insensitive to amino acids (Fig. 7 C), but ΔPX-PLD1 did not localize to the lysosomal area (Fig. 7, A and B). In complete correlation with their differing abilities to translocate to lysosomes, both wt PLD1 and Δloop-PLD1 enhanced amino acid–induced PLD1 lysosomal translocation in an hVps34-dependent fashion.

Figure 6. Amino acids regulate PLD1 translocation to the lysosomal region in an hVps34-dependent fashion. (A) HEK293 cells were transfected with HA-PLD1 followed by serum starvation overnight and amino acid [AA] deprivation for 2 h. Some cells were transduced with lentiviruses expressing hVps34-shRNA [hVps34 knockdown (KD)] and selected with puromycin before transfection of HA-PLD1, whereas others were treated with 10 mM 3-MA for 1 h before amino acid stimulation. Upon amino acid stimulation for 30 min, cells were fixed and immunostained with anti-HA and -LAMP2 antibodies. (B) Cells were treated as in A without transfection and immunostained with anti-mTOR and -LAMP2 antibodies. (C) Cells were transfected with HA-PLD1, treated as in A, and then immunostained with anti-HA and -mTOR antibodies. (A–C) The merged images were pseudocolored as follows: LAMP2 in red and PLD1 in red and mTOR in green (A and B) and PLD1 in red and mTOR in green (C). Enlarged images of the merges are shown in the rightmost columns. Bars, 5 µm; (enlarged images) 0.5 µm. (D) Percentage of LAMP2-colocalized cells among HA- (for PLD1) or mTOR-positive cells was quantified for experiments shown in A and B. Cells with the majority of HA-PLD1 or mTOR signals overlapping with LAMP2 signals were scored as colocalizing. Mean results of three independent experiments are shown with error bars representing SD.
overexpression did not have a significant effect (Fig. 8 B). Hence, hVps34 and the PX domain of PLD1 are important for cell size control, consistent with their roles in regulating mTORC1.

PLD1 and Rag pathways act in parallel to mediate amino acid activation of mTORC1

As both PLD1 and mTORC1 undergo amino acid–stimulated translocation to the lysosomal area, an obvious question is whether the two pathways intersect. We had already found that hVps34 regulates PLD1 translocation without affecting mTOR translocation (Fig. 6), and we wished to address whether Rag and the P18–P14–MP1 Ragulator complex, responsible for mTORC1 lysosomal translocation (Sancak et al., 2008, 2010), are involved in the regulation of PLD1. To that end, we examined the effects of knocking down Rag and P18 on PLD1 activation and translocation. Four Rag proteins (Rag A, B, C, and D) function as heterodimers to regulate mTORC1 translocation (Sancak et al., 2008). We knocked down Rag C and Rag D simultaneously to eliminate all Rag function. As shown in Fig. 9 A, knockdown of Rag, P18, raptor, and hVps34 each drastically blocked amino acid–stimulated S6K1 phosphorylation, whereas ΔPX-PLD1 did not have this effect (Fig. 7 D). Although the Δloop mutant displayed higher PLD activity than the wt protein, it activated S6K1 to a similar degree as the wt, possibly because maximal S6K1 activation in the absence of mitogens was already reached. These observations strongly suggest that the catalytic activity of PLD1 alone is insufficient for mTORC1 activation and that the translocation of PLD1 is necessary for this activation.

Further validating the importance of PLD1 lysosomal translocation is our observation that exogenous PI3P did not induce PLD1 translocation (Fig. S4 A) or S6K1 activation (Fig. S4 B), even though it activated PLD1 to a similar extent as amino acid stimulation (Fig. S4 C). PI3P binding to the PX domain of PLD1 appears to be required for both the catalytic activation and translocation of PLD1, but, although this interaction may be sufficient for the activation of PLD1, translocation of PLD1 most likely has additional requirements yet to be identified.

hVps34 and PLD1 control cell size

To assess the biological significance of the mechanistic connection between hVps34, PLD1, and mTORC1, we considered cell size regulation, a prominent function of mTORC1. Indeed, knockdown of hVps34 and PLD1 each reduced cell size by ~8%, similar to the effects of raptor knockdown and rapamycin treatment (Fig. 8 A). On the other hand, overexpression of wt PLD1 increased cell size by ~6%, whereas ΔPX-PLD1 overexpression did not have a significant effect (Fig. 8 B). Hence, hVps34 and the PX domain of PLD1 are important for cell size control, consistent with their roles in regulating mTORC1.
Sancak et al. (2008) reported that expression of a constitutively active Rag complex activated mTORC1 in the absence of amino acids. We confirmed this observation with coexpression of Rag mutants that represented Rag B–GTP (Q99L) and Rag C–GDP (S75L), which stimulated S6K1 activation in cells deprived of both serum and amino acids (compare first and fifth lanes in Fig. 9 D). However, amino acids further enhanced S6K1 activation in cells expressing constitutively active Rag. Importantly, PLD1 knockdown impaired S6K1 activation induced by Rag both in the presence and absence of amino acids (Fig. 9 D). This observation suggests that the ability of constitutively active Rag to activate mTORC1 is most likely dependent on the basal activity of PLD1, which is present in the lysosomal region in a portion of the cells without amino acid stimulation (Fig. 6 D). Collectively, our data support a model in which the hVps34-PLD1 and Rag pathways act in parallel—both necessary—to activate mTORC1.

Discussion

Although several mechanisms have been described for the activation of the mTORC1 signaling network by amino acids in recent years, gaps have remained in this intricate circuitry. Our present study has revealed a new role of PLD1 in transducing amino acid signals upstream of mTORC1 and filled a gap between hVps34 and mTORC1 in the amino acid–sensing pathway. A direct regulatory relationship is established for hVps34 and PLD1, in that hVps34 activates PLD1 catalytic activity and induces its subcellular translocation through a functional interaction between PI3P and the PX domain in PLD1. With its previously established role in mitogenic signaling, PLD1 now emerges as an integrator of amino acid and mitogen signals upstream of mTORC1. Furthermore, PLD1 translocation to the lysosomal region upon amino acid stimulation, simultaneous to mTOR translocation to the same subcellular locus (Sancak et al., 2008, 2010; Flinn et al., 2010), suggests a unifying mechanism of spatial regulation underlying mTORC1 activation by amino acids (Fig. 10).
early endosomes (Gillooly et al., 2000). However, lower concentrations of PI3P in other subcellular locations could still be physiologically significant. In fact, hVps34 has been found on late endosomes (Stein et al., 2003; Cao et al., 2007) and is known to regulate multivesicular body generation (Futter et al., 2001). Furthermore, a knockdown study has revealed a specific function of hVps34 in the late endosomes in human U-251 glioblastoma cells (Johnson et al., 2006). Our present study identifies PLD1 as a new effector for hVps34-produced PI3P. Although we do not know where exactly the interaction between PLD1 and hVps34 occurs in the cell, it is clear that PLD1 translocation to the late endosomal/lysosomal surface upon amino acid stimulation is dependent on hVps34. However, hVps34 possible in cells in which PLD2 is the predominant PLD isofrom. The involvement of PLD2 in mTORC1 signaling has been reported by others (Chen et al., 2005; Ha et al., 2006; Toschi et al., 2009).

A new effector for hVps34
Among the various cellular functions regulated by hVps34, two major ones are vesicular trafficking and autophagy, involving distinct complexes containing hVps34 (Backer, 2008). Regulation of amino acid–sensing mTOR signaling may be mediated by yet another population of hVps34, although there may exist an interrelationship among these pools of hVps34. PI3P, presumably mediating all hVps34 functions, is found enriched on early endosomes (Gillooly et al., 2000). However, lower concentrations of PI3P in other subcellular locations could still be physiologically significant. In fact, hVps34 has been found on late endosomes (Stein et al., 2003; Cao et al., 2007) and is known to regulate multivesicular body generation (Futter et al., 2001). Furthermore, a knockdown study has revealed a specific function of hVps34 in the late endosomes in human U-251 glioblastoma cells (Johnson et al., 2006). Our present study identifies PLD1 as a new effector for hVps34-produced PI3P. Although we do not know where exactly the interaction between PLD1 and hVps34/IP3P occurs in the cell, it is clear that PLD1 translocation to the late endosomal/lysosomal surface upon amino acid stimulation is dependent on hVps34. However, hVps34
itself appears to be distributed throughout the cytoplasm and the endosomal system without detectable translocation in response to amino acids (unpublished data). Recently, Dall’Armi et al. (2010) reported localization of PLD1 to autophagosomes upon nutrient deprivation, which also appeared to be dependent on hVps34.

Although the PX domain of PLD is highly homologous to those that have been shown to bind phosphoinositides, previous characterization of the PLD1 PX domain has not led to a clear consensus regarding its lipid-binding property and function (Morris, 2007). Depending on the methods used to assess lipid binding, PLD1 PX has been shown to preferentially bind either PI-3,4,5-P3, PI-3,4-P2, or PI3P in vitro (Du et al., 2003). Although not of the highest affinity, PI3P has also been shown to bind the PX domain of PLD1 and activate PLD1 in vitro (Hodgkin et al., 2000; Stahelin et al., 2004). It is conceivable that an intrinsically low affinity between PI3P and PLD could be overcome by a high local concentration of the lipid in the cells, such as when exogenous PI3P was delivered into the cells or when the cells were stimulated by amino acids. The physical interaction between PLD1 and hVps34 may confer proximity advantage and increase the local concentration of PI3P for effective binding and subsequent activation and translocation of PLD1.

Two pathways converging to activate mTORC1 at the late endosomes/lysosomes

It has been shown that amino acids stimulate mTORC1 translocation to the lysosomal region where Rheb presumably resides, and this translocation is mediated by Rag and the P18–P14–MP1 Ragulator complex (Sancak et al., 2008, 2010). Our present study suggests that PLD1 translocation in response to the same signal, through hVps34 and PI3P, occurs independently and in parallel to Rag-mediated mTORC1 translocation (Fig. 10). The translocation of PLD1 to this distinct location of mTORC1 activation further validates the key role of this protein in mediating both mitogenic and amino acid signals to mTORC1 signaling.

Consistent with the model of two independent amino acid–sensing pathways working in parallel (Fig. 10), neither hVps34 overexpression (Flinn et al., 2010) nor PI3P knockdown (Fig. 6 B) affects mTORC1 translocation. Conversely, knockdown of Rag, P18, or raptor has no effect on PLD1 activation or translocation (Fig. 9). The fact that PA alone is not sufficient to activate mTORC1 signaling in the absence of amino acids (Fang et al., 2001) can be readily explained by the parallel requirement of mTORC1 translocation via Rag. On the other hand, PA rescues amino acid activation of mTORC1 from the negative effect of hVps34 knockdown, in full agreement with an amino acids–hVps34-PLD1 pathway. It has been reported that overexpression of a constitutively active Rag dimer or recombinant nptor fused to the membrane-targeting signal of Rheb can override the requirement of amino acid signals for mTORC1 activation (Sancak et al., 2008, 2010), suggesting that the Rag pathway may be sufficient to mediate amino acid activation of mTORC1. However, we have found that amino acids further activate S6K1 in cells overexpressing active Rag and that PLD1 is necessary for Rag activation of S6K1 (Fig. 9 D), validating the requirement of two parallel pathways for the activation of mTORC1 (Fig. 10). In conclusion, the collective evidence we have presented identifies a critical role of the hVps34-PLD1 pathway in amino acid activation of mTORC1 signaling and unveils a clearer view of this nutrient-sensing network of central importance.

Materials and methods

Antibodies and other reagents

The antibodies used were obtained from the following sources: anti-FLAG M2 and anti-P18 from Sigma-Aldrich; anti-Myc (9E10.2) and anti-HA (16B12) from Covance; anti-tubulin and anti-LAMP2 from Abcam; anti-v5 from Invitrogen; and all other antibodies from Cell Signaling Technology. PLD1 antibody was generated by ProteinTech Group, Inc. using a synthetic peptide corresponding to the C-terminal sequence of PLD1. Rapamycin was purchased from LC laboratories. 9,10-3H-oleic acid was obtained from PerkinElmer. PI3P and its polyamine carrier (no. 3) were obtained from Echelon Biosciences Inc. 1,2-dioctanoyl-sn-glycero-3-PA (C8-PA) was obtained from Avanti Polar Lipids, Inc. All other reagents were obtained from Sigma-Aldrich.

Plasmids

The following plasmids have been previously reported: Myc-S6K1, HA-S6K1, and HA-PLD1 (Fang et al., 2003); wt and kinase-dead [D743I and N748I] hVps15 (Yan et al., 2009); all PLD1 mutants (Sung et al., 1999; Du et al., 2003); and HA-PLD2 (Colley et al., 1997). HA-OST-RagB-Q99L and HA-OST-RagC-S75L were obtained from Addgene (Sancak et al., 2008). The RNAi-resistant mutants of wt and kinase-dead hVps34 were created by standard site mutagenesis methods, changing nucleotides 2,436–2,438 from 5-TATTCT-3 to 5-TATTCC-3. The EGFP-2xFYVE construct was a gift from the Stenmark laboratory at the Norwegian Radium Hospital (Gillooly et al., 2000).

Cell culture and transfection

HEK293 cells were grown in DME containing 10% FBS at 37°C with 5% CO2. Transient transfections were performed with PolyFect (QIAGEN) following the manufacturer’s recommendations. Serum starvation was performed by incubating cells in serum-free DME at 37°C with 5% CO2 overnight. Amino acid or Leu starvation was achieved by incubation in amino acid– or Leu-free DME [HyClone] for 2 h after serum starvation, respectively. Amino acid or Leu stimulation was performed by incubating cells in regular DME for 30 min. Detailed conditions for serum, insulin, and PA stimulation are described in the figure legends where applicable. C8-PA vesicles were freshly made for each experiment as previously described (Yoon et al., 2011). In brief, C8-PA in chloroform was dried and reconstituted into C8-PA vesicles were freshly made for each experiment as previously described. Upon amino acid stimulation, two parallel pathways converge on the lysosome to activate mTORC1.

Figure 10. A proposed model for amino acid–sensing mTORC1 signaling. Upon amino acid stimulation, two parallel pathways converge on the lysosome to activate mTORC1.
followed by sonication in a water bath sonicator (at 600 V, 80 kilocycles, and 0.5 A; G112SP; Laboratory Supplies Co., Inc.) for 5 min. All other cell lines were treated in the same manner as for HEK293 cells, except that DME containing 1g/L glucose was used for C2C12 and HepG2 cells.

**Lentivirus-mediated RNAi**
All shRNAs were in the pLKO.1-puro vector from The RNAi Consortium (TRC, Sigma-Aldrich). The following shRNA clones for hVps34 were obtained from Sigma-Aldrich: hVps34-1 (TRCN0000037779) and hVps34-2 (TRCN0000037779). The shRNAs for human PLD1 (TRCN0000010011 and TRCN0000010572), mouse PLD1 (TRCN0000076820), and human PLD2 (TRCN0000051149 and TRCN0000051150) were previously reported (Sun et al., 2008). shRNAs for the following genes were obtained from Sigma-Aldrich based on published information: Rag C (TRCN0000072874), Rag D (TRCN0000059533), and P18 (TRCN0000026326; Sancak et al., 2008, 2010). Raptor shRNA and a negative control shRNA (containing a scrambled sequence in the hairpin) were obtained from Addgene (Sarbassov et al., 2005b). Lentivirus packaging was performed by cotransfecting plKO-shRNA, pCMV-dR8.91, and pCMV-NSP into 293T cells using TransIT-LT1 (Minic Bio LLC) at 0.5, 0.45, and 0.05 μg, respectively, for 1 well in a 6-well plate. Media containing viruses were collected 48 h after transfection. Cells were infected with the viruses in the presence of 6 mg/mL polybrene for 24 h and were then subjected to selection by 1.5 μg/mL puromycin for 72 h.

**Cell lysis, immunoprecipitation, and Western analysis**
Cells were rinsed with ice-cold PBS and lysed in ice-cold lysis buffer (Villegas-Bach et al., 1999) with 1x protease inhibitor cocktail (Sigma-Aldrich). Immunoprecipitation was performed with the lysates at 4°C, and the beads were washed and rinsed with PBS. Protein samples were boiled in SDS sample buffer and subjected to Western analysis using HRP-conjugated secondary antibodies detected with chemiluminescence reagent (Western Lightning Plus; PerkinElmer).

**In vivo PLD assay**
Cells were transfected in a transphosphatidylation assay as previously described (Sun et al., 2008). In brief, HEK293 cells were labeled with 3H-oleic acid for 1 d and subjected to various treatments as previously described (Sun et al., 2008). In brief, HEK293 cells were lyzed, and lipids were extracted and analyzed by thin-layer chromatography. Precipitation was performed with the lysates at 4°C, and the beads were washed with PBS. Protein samples were boiled in SDS sample buffer and subjected to Western analysis using HRP-conjugated secondary antibodies detected with chemiluminescence reagent (Western Lightning Plus; PerkinElmer).

**Intracellular delivery of phospholipidinositides**
Unlabeled phospholipid carrier 3 (Echelon Biosciences Inc.) and PI3P or PI was mixed at a 1:1 molar ratio (200 μM final concentration) in a Pyrex tube. After brief vortexing and bath sonication, the complex was incubated at room temperature for 15 min and then added to cells at a final concentration of 15 μM.

**Immunofluorescence imaging**
HEK293 cells cultured on poly-lysine-coated glass coverslips were transfected and treated as indicated in the figure legends followed by fixation in 3.7% PFA and permeabilization with 0.1% Triton X-100. Incubation with various primary antibodies was performed in 3% BSA/PBS at 4°C overnight followed by incubation with Alexa Fluor anti–mouse 594 and –rabbit 488 antibodies in 3% BSA/PBS for 30 min at room temperature. A personal deconvolution microscope system (DeltaVision; Applied Precision) was used with antibodies in 3% BSA/PBS for 30 min at room temperature. A personal deconvolution microscope system (DeltaVision; Applied Precision) was used with antibodies in 3% BSA/PBS for 30 min at room temperature. A personal deconvolution microscope system (DeltaVision; Applied Precision) was used with antibodies in 3% BSA/PBS for 30 min at room temperature.

**Cell size measurement**
Cells were trypsinized and resuspended in PBS, and cell size was measured using a flow cytometer (LSRII; BD). 30,000 cells were analyzed for each sample to obtain the parameter of median forward scatter-height.

**Online supplemental material**
Fig. S1 shows that PLD2 is not involved in amino acid activation of mTORC1. Fig. S2 shows that recombinant PLD2 localization is not regulated by amino acids. Fig. S3 shows that the catalytic activity of PLD1 is not necessary for amino acid–induced translocation. Fig. S4 shows that exogenous PI3P stimulates PLD1 activity but not PLD1 translocation or S6K1 phosphorylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107033/DC1.