PLD regulates myoblast differentiation through the mTOR-IGF2 pathway

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Summary

A mammalian target of rapamycin (mTOR) pathway is essential for the differentiation of cultured skeletal myoblasts in response to growth factor withdrawal. Previously, phospholipase D (PLD) has been found to play a role in cell growth regulation and mitogenic activation of mTOR signaling. However, a role for PLD in the autocrine regulation of myoblast differentiation is not known. Here we show that upon induction of differentiation in mouse C2C12 satellite cells, the expression of both PLD1 and PLD2 is upregulated. C2C12 differentiation is markedly inhibited by 1-butanol, an inhibitor of the PLD-catalyzed transphosphatidylation reaction, and also by the knockdown of PLD1, but not PLD2. Further investigation has revealed that PLD1 is unlikely to regulate myogenesis through modulation of the actin cytoskeleton as previously suggested. Instead, PLD1 positively regulates mTOR signaling leading to the production of IGF2, an autocrine factor instrumental for the initiation of satellite cell differentiation. Furthermore, exogenous IGF2 fully rescues the differentiation defect resulting from PLD1 knockdown. Hence, PLD1 is critically involved in skeletal myogenesis by regulating the mTOR-IGF2 pathway.

Key words: mTOR, Myogenesis, Phospholipase D (PLD)

Introduction

The process of skeletal muscle differentiation is characterized by mononucleated myoblasts exiting the cell cycle and fusing to form multinucleated myotubes. The MyoD and myocyte enhancer factor-2 transcription factor families are mainly responsible for regulating this process of cell cycle withdrawal and initiation of differentiation (Olson et al., 1995; Weintraub, 1993). Most mitogens promote myoblast proliferation and inhibit differentiation, whereas insulin and insulin-like growth factors (IGF1 and IGF2) stimulate both processes in muscle cell cultures (Florini et al., 1991a; Perry and Rudnicki, 2000). IGF1 and IGF2 are critically involved in skeletal muscle development and adult muscle regeneration and hypertrophy (Barton-Davis et al., 1999; Florini et al., 1991a), which require satellite cell activation and differentiation (Barton-Davis et al., 1999; Rosenblatt et al., 1994). In cultured myoblasts, growth factor deprivation often initiates the differentiation program owing to the induction of IGF2 (Florini et al., 1991b; Tollefsen et al., 1989) and subsequent activation of the IGF1 receptor and a major downstream pathway mediated by PI3K and Akt (Perry and Rudnicki, 2000).

The cellular target of the bacterial macrolide rapamycin, mTOR, is a Ser/Thr kinase that functions as a master regulator of cell growth, proliferation, and various types of cellular differentiation, by sensing nutrient availability and cellular energy levels (Erbay et al., 2005; Hay and Sonenberg, 2004). A critical role of mTOR in the regulation of myoblast differentiation has been suggested by the inhibitory effects of rapamycin on the differentiation of various myoblast cell cultures (Conejo et al., 2001; Coolican et al., 1997; Cuenda and Cohen, 1999; Erbay and Chen, 2001) and validated by the ability of a rapamycin-resistant recombinant mTOR to fully rescue C2C12 differentiation in the presence of rapamycin (Erbay and Chen, 2001; Shu et al., 2002). Interestingly, neither the kinase activity of mTOR, nor its downstream effectors S6K1 and 4E-BP1 – which are well characterized regulators of protein synthesis – are required for the myogenic function of mTOR at the initiation of C2C12 differentiation (Erbay and Chen, 2001; Erbay et al., 2003). Instead, mTOR regulates the early stage of myogenesis by controlling nutrient-dependent IGF2 production at the transcriptional level via an unidentified mechanism (Erbay et al., 2003). However, neither the immediate downstream targets nor the upstream regulators are known in this non-canonical mTOR pathway.

Phospholipase D (PLD) hydrolyzes phosphatidylcholine to yield choline and phosphatidic acid in response to a variety of extracellular stimuli (Exton, 1999; Frohman et al., 1999). PLD and phosphatidic acid play important roles in cellular signaling, in which phosphatidic acid acts as a second messenger. Whereas PLD has been traditionally associated with vesicular functions (Liscovitch et al., 2000) and cytoskeleton reorganization (Colley et al., 1997; Kam and Exton, 2001), PLD has long been implicated in the regulation of cell growth and proliferation (Foster and Xu, 2003) and has been linked to mTOR signaling (Chen and Fang, 2002; Foster, 2007). Phosphatidic acid mediates mitogenic activation of the mTOR pathway (Fang et al., 2001), and PLD is an upstream regulator of mTOR in a variety of cell types under growth and proliferation conditions (Foster, 2007). There are two mammalian isoforms of PLD: PLD1 has a low basal activity and can be activated by various stimuli (Exton, 1999) whereas PLD2 displays a higher basal activity (Colley et al., 1997). Yet, the isoform responsible for regulating mitogenic mTOR signaling is still a topic of debate (Fang et al., 2003; Ha et al., 2006).

A role for PLD in vasopressin-stimulated rat L6 myoblast differentiation has been suggested (Naro et al., 1997) and it has been reported that PLD acts through remodeling of the actin

cytoskeleton (Komati et al., 2005). Notably, a potential role of PLD in the autocrine regulation of satellite cell differentiation upon growth factor withdrawal has not been examined, and whether, and how, PLD is involved in mTOR signaling in myogenic differentiation is not known. In the current study, we investigate the involvement of PLD in mouse C2C12 satellite cell differentiation, and probe a mechanistic relationship between PLD and the mTOR pathway in myogenesis.

Results

PLD is upregulated during C2C12 differentiation

To examine the role of PLD in myogenesis, we first assessed PLD expression in the mouse satellite cell line C2C12 upon differentiation. C2C12 myoblasts grown to 100% confluence were induced to differentiate by serum withdrawal (2% horse serum). Typically, cell fusion and formation of small myotubes were evident 2 days after induction, and myotubes were fully formed in 3 days. After 1 day of differentiation the mRNA levels of both *Pld1* and *Pld2* increased ~twofold, and they stayed elevated throughout the differentiation (Fig. 1A). The protein level of PLD1 also increased during C2C12 differentiation, as detected by western blotting (Fig. 1B). We were not able to detect PLD2 protein in western blots with the available antibodies, probably owing to low expression levels of PLD2 and/or poor affinities of the antibodies. Furthermore, we found that the total cellular PLD activity rose 2.6-fold as the differentiation of C2C12 cells proceeded. This correlation of differentiation and upregulation of PLD suggested that PLD might be involved in myogenic differentiation of C2C12 cells.

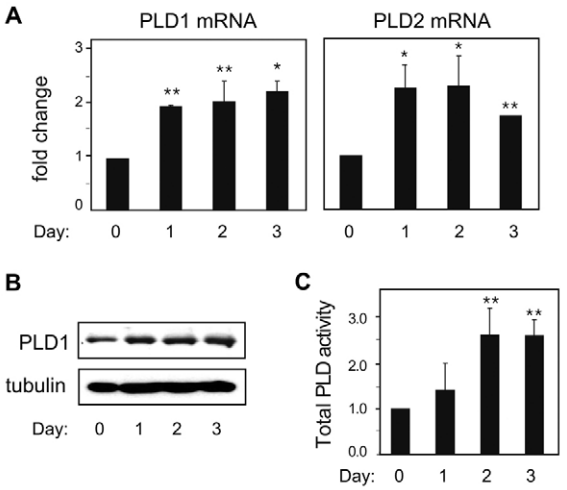

Fig. 1. PLD is upregulated during C2C12 differentiation. Confluent C2C12 cells were induced to differentiate by serum withdrawal. Cells were lysed every 24 hours, and the lysates were subjected to (A) quantitative RT-PCR and (B) western analysis. (C) In vivo transphosphatidylation assays were performed with differentiating C2C12 cells. The average results of three independent experiments are shown, with error bars representing s.d. Student's *t*-test was performed to compare the data from day 1 to 3 with that of day 0. **P*<0.05; ***P*<0.01.

1-butanol inhibits C2C12 differentiation

To further address the role of PLD in myogenesis, we used 1-butanol, a primary alcohol that prevents the formation of the PLD enzymatic product phosphatidic acid. As shown in Fig. 2A, treatment by 0.5% 1-butanol drastically diminished the degree of C2C12 differentiation, as evaluated by visualizing MHC-positive myotubes. DAPI staining was performed to allow assessment of cell density and calculation of fusion index (see below). As a negative control, 0.5% 2-butanol – a secondary alcohol that does not effectively interfere with PLD-catalyzed reactions – did not have any obvious effect on differentiation. 1-butanol treatment modestly (but nevertheless noticeably) reduced the total amount of nuclei by day 3, indicating that this inhibitor may affect cell proliferation and/or survival. It was thus important that the relative differentiation potential be quantified by the fusion index, defined as the ratio of nuclei number in myocytes with two or more nuclei versus the total number of nuclei. Treatment with 1-butanol, but not 2-butanol, drastically reduced the fusion index (Fig. 2B). We also examined the expression of two differentiation markers – the early marker myogenin and the contractile protein MHC – by western analysis. Treatment by 1-butanol, but not 2-butanol, significantly reduced the expression of both myogenin and MHC, but not tubulin – the loading control (Fig. 2C). As expected, rapamycin severely blocked myotube formation (Fig. 2A,B) and the expression of myogenin and MHC (Fig. 2C). These results implicate PLD as a critical regulator in myogenesis, possibly at an early step of the process.

PLD1, but not PLD2, is required for C2C12 differentiation

The potentially nonspecific cytotoxicity of 1-butanol called for further examination of the role of PLD in myogenic differentiation, so we investigated the effects of PLD knockdown by lentivirus-

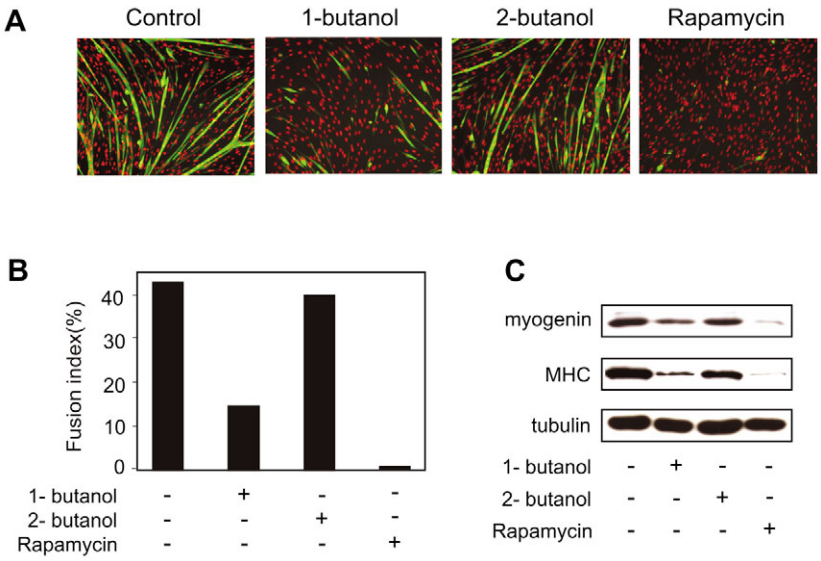

Fig. 2. 1-butanol inhibits C2C12 differentiation. C2C12 myoblasts were induced to differentiate for 3 days in the presence or absence of 0.5% 1-butanol, 0.5% 2-butanol, or 50 nM rapamycin. (A) The cells were immunostained for MHC (green) and DAPI (red). (B) Fusion index of cells shown in A. (C) Cells were lysed and subjected to western analysis for MHC, myogenin and tubulin. Three independent experiments were performed with similar outcomes; the result of a representative experiment is shown.

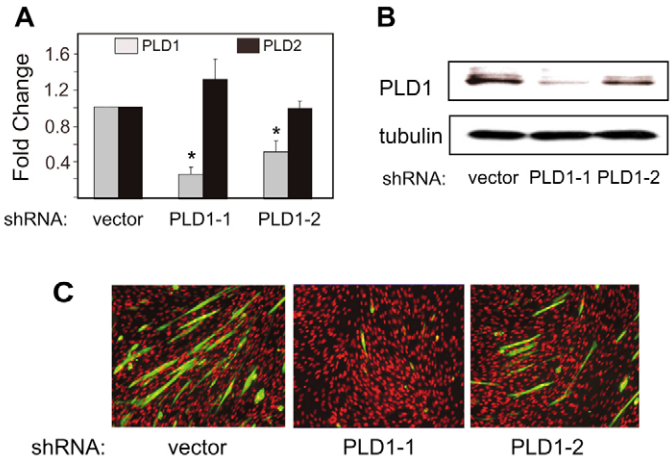

Fig. 3. Knockdown of PLD1 leads to decreased myogenic differentiation. C2C12 cells were infected with lentiviruses expressing two different *Pld1* shRNAs or the empty vector, selected by puromycin for 3 days, and then subjected to analysis by (A) quantitative RT-PCR to measure levels of *Pld1* and *Pld2* mRNA, and (B) western blotting for PLD1. (C) The shRNA-expressing cells were grown to confluence and induced to differentiate for 3 days, followed by immunostaining for MHC. Three independent experiments were performed in each case with similar outcomes, and representative data are shown, except for A, where the average data of all experiments are shown with the error bars representing the s.d. Student's *t*-test was performed to compare cells infected with *Pld1* shRNA viruses and empty vector virus. $*P \leqslant 0.01$.

delivered shRNA. The experiments were performed with two independent shRNAs for each *Pld* gene, and either the empty pLKO vector or a scrambled hairpin sequence was used as a negative control. shRNAs targeting *Pld1* specifically reduced the mRNA levels of *Pld1* by 70% and 50% without any significant effect on *Pld2* mRNA (Fig. 3A), and the protein level of PLD1 decreased correspondingly (Fig. 3B). When myoblasts expressing these shRNAs were induced to differentiate, a significant reduction of myotube formation in the PLD1-knockdown cells was observed (Fig. 3C; the fusion indexes are presented in Fig. 7C). The different degrees of differentiation inhibition by the two independent shRNA constructs correlated closely with their capacities to reduce PLD1 levels, confirming the specificity of the knockdown. The residual differentiation in the shRNA-expressing cells could be due to the incomplete removal of the PLD1 protein by knockdown. Thus, PLD1 appears to be essential for C2C12 differentiation.

Two shRNAs targeting *Pld2* were also characterized, and they reduced the *Pld2* mRNA level by 40-50% without affecting the *Pld1* mRNA (Fig. 4A). Of note, the degree of mRNA knockdown by these shRNAs was comparable to that by one of the *Pld1* shRNAs described above. Because we were unable to detect the endogenous PLD2 protein from C2C12 cells with currently available antibodies, we further characterized the knockdown by examining cellular PLD activity. PLD2 is known to display high basal activity in cells, whereas PLD1 activity is basally low until activated by a variety of stimuli including phorbol 12-myristate 13-acetate (PMA). Indeed, we observed that the expression of *Pld2* shRNAs led to a decrease in the total basal PLD activity to 50-60%, without inhibiting PMA-stimulated PLD activity (Fig. 4B, compare shRNA PLD2-1 and PLD2-2 to scramble). These results confirm the specific knockdown of PLD2 by these shRNAs. However, no effect on C2C12 differentiation was observed accompanying the knockdown (Fig. 4C). Although we cannot definitively conclude that PLD2 is dispensable for myogenesis, cumulatively our data suggest that PLD1 plays a more critical role in the regulation of myogenesis.

PLD1 does not regulate actin cytoskeleton reorganization in C2C12 cells

It was reported that PLD1 is involved in the arginine-vasopressin (AVP)-induced myogenic differentiation of rat L6 cells through remodeling of the actin cytoskeleton – specifically, stress fiber formation (Komati et al., 2005). So, we examined whether PLD1 was involved in actin stress fiber formation during C2C12 differentiation. Upon the induction of differentiation by serum withdrawal for 12 hours or 24 hours, an increase in actin fiber structure, mostly stress fibers, was evident as revealed by immunostaining with Rhodamine-conjugated phalloidin (Fig. 5). C2C12 cells expressing PLD1 shRNAs displayed a similar cytoskeletal change, indistinguishable from the cells expressing the scrambled shRNA (Fig. 5). We also examined the effect of 1-butanol, which would inhibit PLD more completely than the knockdown constructs. Despite the potentially general cytotoxicity in addition to inhibition of all

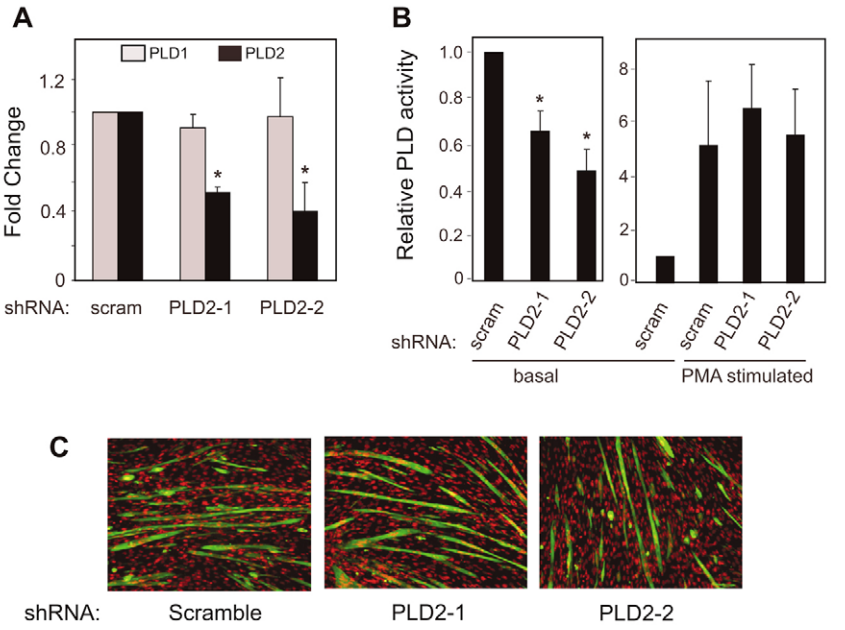

Fig. 4. Knockdown of PLD2 does not affect C2C12 differentiation. (A) C2C12 cells were infected with lentiviruses expressing two different *Pld2* shRNAs or a scrambled hairpin sequence (Scram) as negative control, selected by puromycin for 3 days, and then subjected to analysis by quantitative RT-PCR. (B) Cells treated as above were subjected to in vivo PLD assay with or without stimulation by 100 nM PMA for 30 minutes. For A and B, the average results of three independent experiments are shown, with error bars representing the s.d. Student's *t*-test was performed to compare cells infected with *Pld2* shRNA viruses and scrambled virus. $*P \leqslant 0.01$. (C) The shRNA-expressing cells were grown to confluence, induced to differentiate for 3 days, and immunostained for MHC (green) and DAPI (red). Similar outcomes were observed for three independent experiments, and a representative set of data is shown.

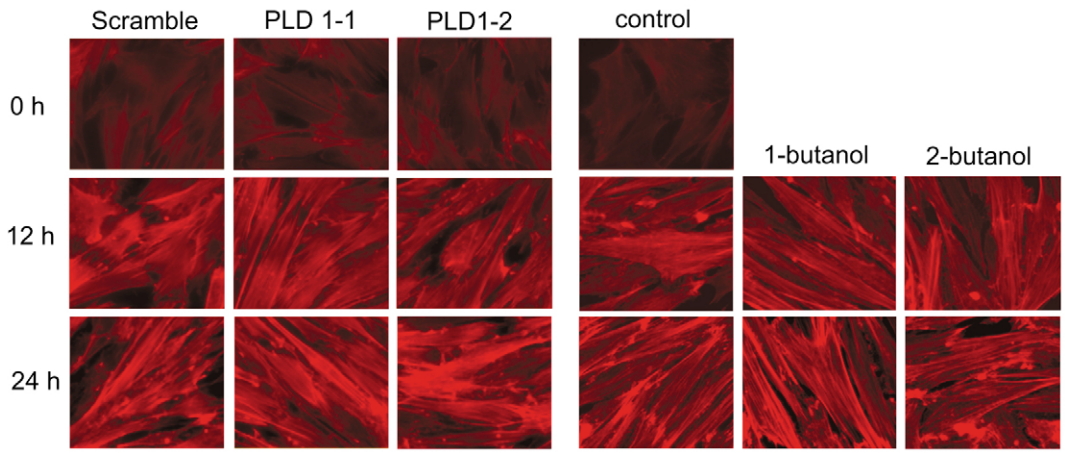

Fig. 5. PLD1 does not function through the actin cytoskeleton in C2C12 differentiation. C2C12 cells were infected with lentiviruses expressing two different *Pld1* shRNAs or a scrambled hairpin sequence as a negative control, selected with puromycin for 3 days and induced to differentiate. Separately, the non-infected cells were subjected to 0.5% 1- or 2-butanol treatment during differentiation. At 0, 12 hours and 24 hours differentiation, the cells were fixed and labeled with Rhodamine-phalloidin. The samples to be compared were stained simultaneously, with image capture performed with an identical exposure time.

PLD isoforms, 1-butanol displayed no detectable effect on the actin stress fiber formation induced by serum withdrawal for 12 hours and 24 hours (Fig. 5). Hence, it is highly likely that PLD1 regulates C2C12 differentiation through a mechanism independent of the modulation of the actin cytoskeleton.

PLD1 regulates mTOR signaling and the autocrine production of IGF2 during C2C12 differentiation

In the context of cell growth (cell size) regulation, PLD1, via its product phosphatidic acid, mediates mitogenic activation of the mTOR signaling pathway (Fang et al., 2003; Fang et al., 2001). PLD and phosphatidic acid have also been linked to mechanical stimulation of mTOR signaling in skeletal muscles (Hornberger et al., 2006). However, the relationship between PLD and mTOR in myoblast differentiation had not been examined before. We used the activation of S6K1, a downstream effector of mTOR in various cellular contexts, as a measure of mTOR activity. The expression of *Pld1* shRNA suppressed S6K1 activity, as measured by phosphorylation of S6K1 on T389, at day 3 following the induction of differentiation by serum withdrawal (Fig. 6A). The decrease of T389-*P* correlated closely with the degree of PLD1 reduction by the two independent shRNA constructs, again validating the specificity of the knockdown effect. It should be noted that despite an increase in S6K1 activity during myogenic differentiation, S6K1 is not required for mTOR regulation of the initiation of differentiation (Erbay and Chen, 2001; Erbay et al., 2003). Nevertheless, S6K1 activity served here as an indicator of mTOR signaling. Thus, PLD1 acts upstream of mTOR during myogenesis.

To ascertain that the myogenic function of PLD is through the mTOR pathway, we considered our previous finding that mTOR signaling regulates the transcription of IGF2, an autocrine factor responsible for initiating differentiation in C2C12 cells (Erbay et al., 2003). If PLD1 is indeed an upstream regulator of mTOR signaling in myogenesis, one would expect that the production of IGF2 requires PLD1. To test this possibility, we first examined the effect of PLD1 knockdown on IGF2 expression during C2C12 differentiation. Indeed, the expression of two independent shRNAs targeting *Pld1* led to decreased *Igf2* mRNA levels 3 days after the induction of differentiation (Fig. 6B). 1-butanol treatment had a more dramatic effect on *Igf2* mRNA, as would be expected considering the incomplete removal of the PLD1 protein by knockdown. As we reported before, rapamycin effectively blocked *Igf2* mRNA expression (Fig. 6B). In addition, we found that the IGF2 protein level in the medium during differentiation was also

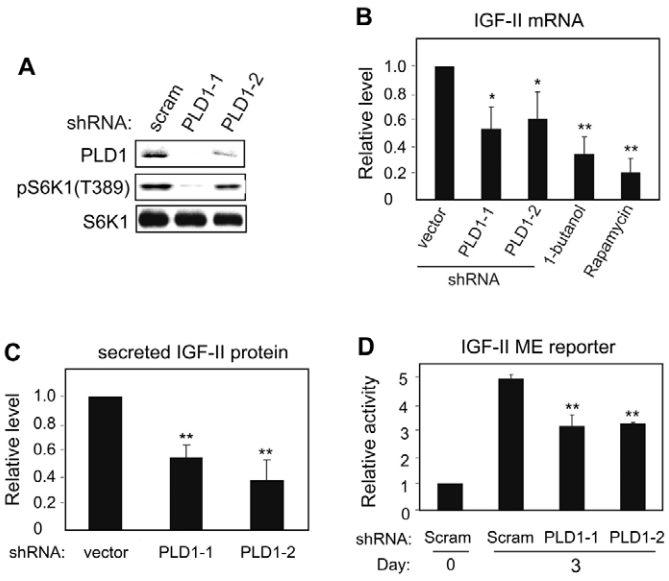

Fig. 6. PLD1 regulates the mTOR-IGF2 pathway during C2C12 differentiation. (A) C2C12 cells were infected with lentiviruses expressing two different *Pld1* shRNAs, the empty vector or a scrambled hairpin sequence (Scram), selected with puromycin, and then induced to differentiate for 3 days. The cell lysates were analyzed by western blotting. (B) Total RNA was extracted from cells expressing *Pld1* shRNAs or treated with 0.5% 1-butanol or 50 nM rapamycin, and *Igf2* mRNA levels were measured by quantitative RT-PCR. (C) IGF2 protein levels in the conditioned media from cells in A were analyzed by ELISA. (D) Cells stably expressing H19-luc-ME were infected with lentiviruses as above, induced to differentiate for 3 days, and then subjected to luciferase assays. For B-D, the average results of three independent experiments are shown, with error bars representing the s.d. Student's *t*-test was performed to compare PLD1 shRNA with the negative control (vector or Scram). **P*<0.05; ***P*<0.01.

significantly reduced accompanying the knockdown of PLD1 (Fig. 6C).

Since mTOR signaling has been shown to regulate the transcription of *Igf2* through a muscle-specific enhancer ('ME') (Erbay et al., 2003), we examined the effect of knocking down PLD1 on the activity of ME measured using a luciferase reporter. C2C12 cell pools stably expressing the ME-luciferase reporter were established, and then *Pld1* shRNAs or scrambled shRNA were introduced via lentiviral infection. As shown in Fig. 6D, the reporter activity increased during the transition from myoblast to myotubes as expected, and knockdown of PLD1 significantly suppressed the reporter activity. Again, this suppression was not complete, and it could be explained by the reduction, rather than elimination, of the endogenous PLD1 protein. Taken together, these results suggest that PLD1 probably exerts its myogenic function by controlling mTOR-dependent IGF2 expression, a critical step in the initiation of the differentiation program.

PLD1 regulates myogenesis through IGF2 production

The production of IGF2 in C2C12 cells leads to autocrine activation of the IGF1 receptor, and subsequent PI3K-Akt signaling (Coolican et al., 1997; Kaliman et al., 1998). Previously, we have shown that Akt activation is both necessary and sufficient to mediate the myogenic function of mTOR via the IGF2 pathway (Erbay et al., 2003). To further validate PLD1 as an upstream regulator of the mTOR-IGF2 pathway, we explored the role of PLD1 in IGF2-Akt signaling during myogenesis. First we evaluated the effect of PLD1 knockdown on Akt activation using both Ser473 and Thr308 phosphorylation as readouts. As shown in Fig. 7A, PLD1 knockdown resulted in significant decreases in the phosphorylation of both Akt sites during C2C12 differentiation. Importantly, the addition of exogenous IGF2 fully rescued Akt phosphorylation in the presence of PLD1 knockdown, confirming that PLD1 most likely acts upstream of IGF2 to eventually impact Akt phosphorylation.

To confirm that IGF2 plays a critical role in mediating PLD1 function during myogenesis, we performed IGF2 rescue experiments, where we treated C2C12 cells expressing *Pld1* shRNAs with exogenous IGF2 and assessed the cell differentiation potential. Strikingly, the negative effect of PLD1 knockdown on myotube formation was completely reversed by the addition of IGF2 (Fig. 7B), as confirmed by the fusion indices (Fig. 7C). Exogenous IGF2 also rescued myotube formation from 1-butanol inhibition (data not shown). Thus, we conclude that PLD1 exerts its myogenic function by regulating the mTOR-IGF2 pathway during myoblast differentiation (Fig. 8).

Discussion

The signaling events that orchestrate the ordered multi-stage process of skeletal myogenesis have started to be revealed in recent research. In the present study, we investigate the potential role of PLD as an upstream signaling molecule in the regulation of myogenic differentiation. We found that the expression levels of both *Pld1* and *Pld2*, as well as total cellular PLD activity, were elevated during C2C12 cell differentiation (Fig. 1), and that treatment with a primary alcohol drastically inhibited the differentiation of the cells at an early stage (Fig. 2). Definitive evidence for the requirement of PLD in myogenesis came from the observation that knockdown of PLD1 by RNAi significantly diminished the myogenic capacity of C2C12 cells (Fig. 3).

It has been reported that PLD1 is involved in the AVP-stimulated myogenic differentiation of L6 rat skeletal myoblasts (Komati et al., 2005; Naro et al., 1997), and that ceramide is a negative regulator in this process upstream of PLD1 (Mebarek et al., 2007). A potentially significant difference between our study and the previously reported ones is that the C2C12 satellite cells undergo differentiation upon growth factor deprivation, in the absence of any hormonal stimulation, which mirrors the myogenic process in normal muscle development and regeneration. Indeed, this difference is reflected by the apparently distinct molecular mechanisms in these two systems. AVP induces an acute actin cytoskeleton rearrangement leading to stress fiber formation in L6 myoblasts, where PLD1 is proposed to regulate differentiation through remodeling of actin

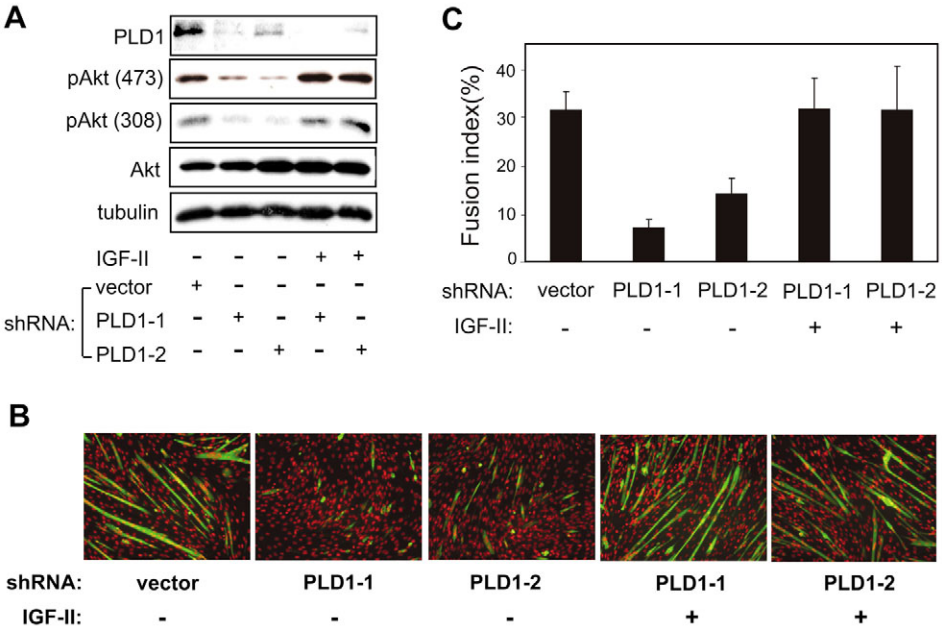

Fig. 7. PLD1 regulates myogenesis through IGF2 production. C2C12 cells were infected with lentiviruses expressing various shRNAs, selected with puromycin, and then induced to differentiate for 3 days with or without exogenous IGF2 (300 ng/ml), followed by (A) western analysis of the cell lysates, or (B) immunostaining of MHC (green) and DAPI (red). (C) The average results of fusion index calculated from three independent experiments in B. Error bars represent s.d.

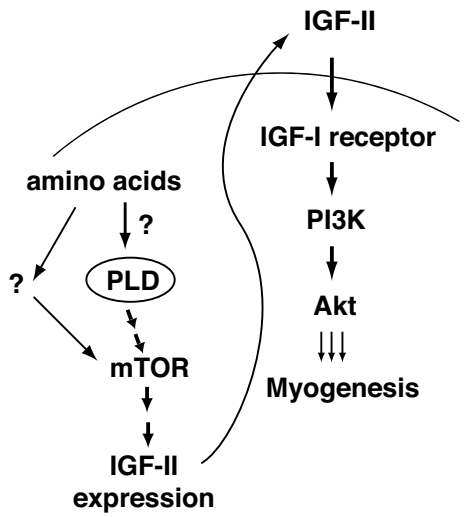

Fig. 8. A proposed model: PLD regulates skeletal myogenesis by controlling the amino-acid-sensing mTOR-IGF2 pathway.

cytoskeleton in a RhoA-dependent manner (Komati et al., 2005). By contrast, although increased actin stress fiber formation was also observed during C2C12 differentiation, neither PLD1 knockdown nor 1-butanol treatment had any discernible effect on the actin cytoskeleton (Fig. 5). Notably, Su et al. reported that RhoA-mediated PLD1 signaling is not required for actin cytoskeletal reorganization in HeLa and CHO cells (Su et al., 2006). Moreover, the activity of RhoA was found to decrease during C2C12 differentiation, and constitutively active RhoA inhibited differentiation (Lim et al., 2007; Nishiyama et al., 2004). Thus, a RhoA-PLD1 pathway is unlikely to play a major (if any) role, and the actin cytoskeleton may not be the target of PLD1 in myogenic differentiation of the satellite cells.

IGF2 is thought to play a pivotal role in the initiation of differentiation of C2C12 cells. Growth factor deprivation induces the production of IGF2 in these cells, which in turn initiates the differentiation program by activating IGF1 receptor signaling in an autocrine fashion (Florini et al., 1991b; Tollefsen et al., 1989). IGF2 expression during skeletal muscle differentiation is regulated at the transcriptional level (Kou and Rotwein, 1993), and previously we have reported that an mTOR pathway regulates nutrient-dependent transcription of IGF2 during the transition from myoblasts to myotubes in C2C12 cultures (Erbay et al., 2003). A link between PLD and mTOR is well established in the context of cell growth and proliferation (Foster, 2007), with phosphatidic acid acting as an upstream regulator of the mitogenic activation of mTOR signaling (Fang et al., 2001). However, the involvement of PLD in myogenic mTOR signaling has not been examined before, and could not be assumed, owing to the significant difference in mTOR regulatory mechanisms between mitogenesis and myogenesis. Importantly, we found that knockdown of PLD1 impaired mTOR activity (Fig. 6A), and diminished the mRNA and protein levels of IGF2 as well as the activity of a muscle enhancer of the *Igf2* gene previously found to be regulated by mTOR (Fig. 6B-D). Exogenous IGF2 fully rescued differentiation from the negative impact of PLD1 knockdown (Fig. 7B,C), suggesting that IGF2 production is sufficient to mediate the myogenic function of PLD1. Knockdown of PLD1 also inhibited Akt phosphorylation and exogenous IGF2 reversed this effect (Fig. 7A), which is consistent with PLD1 being upstream of IGF2 production, as PI3K-Akt mediates signaling downstream of the IGF1 receptor binding to IGF2. Although it cannot be ruled out that PLD1 may modulate the transcription of IGF2 via an unidentified mechanism, the simplest model to explain our observations is one where PLD1 regulates IGF2 production through mTOR (Fig. 8). Since IGF2 expression is regulated by an amino-acid-sensing mTOR pathway during myogenesis, PLD1 could potentially be a mediator of the amino acid signal upstream of mTOR, although it would be equally likely that amino acids act in parallel with PLD1 to impinge on mTOR. The regulation of PLD1 by upstream signals during myogenesis should certainly be investigated in future studies.

In contrast to the regulation of cell growth where the kinase activity of mTOR is essential and S6K1 and 4E-BP1 are key downstream effectors, mTOR signaling in the regulation of *Igf2* expression and initiation of differentiation is not dependent on its kinase activity, nor on S6K1–4E-BP1 activity (Erbay and Chen, 2001; Erbay et al., 2003). Thus, it is interesting that PLD is an upstream regulator of mTOR in both pathways, suggesting that the PLD-mTOR link is not limited to mitogenic signaling or the canonical mTOR-S6K1 pathway. Although the mechanism for phosphatidic acid regulation of mTOR remains unknown, the involvement of PLD in the regulation of a kinase-independent mTOR function further supports the idea that phosphatidic acid is unlikely to modulate the catalytic activity of mTOR (Fang et al., 2001).

Notably, mTOR is also a major regulator of load-induced growth of skeletal muscle (Bodine et al., 2001; Hornberger et al., 2004), and mechanical stimulation activates mTOR signaling through a PLD-dependent phosphatidic acid increase in ex vivo skeletal muscles (Hornberger et al., 2006). Hornberger et al. observed localization of both PLD1 and PLD2 to the z band of skeletal muscle, implying that the two isoforms may play redundant functions in mechanical force transmission (Hornberger et al., 2006). The physiological context is apparently a determinant for the isoform specificity, as our data suggest that PLD1 and not PLD2 is the major contributor in the regulation of C2C12 differentiation. This is reminiscent of our previous observation that PLD1 was required for the mitogenic activation of mTOR signaling and cell size regulation (Fang et al., 2003), although it contradicts a recent report that the knockdown of PLD2, but not PLD1, inhibits S6K1 activation in similar cells (Ha et al., 2006). Obviously, the differential regulation and function of the two isoforms of PLD in mTOR signaling remains an intriguing question.

PLD has been implicated in the regulation of several other types of cellular differentiation. For instance, the activity and expression of PLD increase upon maturation and differentiation of human myeloid cells (El Marjou et al., 2000); upregulation of PLD1 and PLD2 expression is found during granulocytic differentiation of HL60 cells (Nakashima et al., 1998); elevated levels of PLD transcripts have been observed during differentiation of rat C6 glioma cells (Yoshimura et al., 1996), keratinocytes (Griner et al., 1999) and decidualization of human endometrial stromal cells (Yoon et al., 2007). The molecular pathways through which PLD influences these differentiation processes have not been elucidated. PLD interaction with mTOR signaling in these processes warrants future investigation.

Materials and Methods

Antibodies and other reagents

Antibodies against S6K1, pS6K1 (Thr389), Akt, pAkt (Ser473; Thr308), and tubulin were obtained from Cell Signaling Technology. All secondary antibodies were from Jackson ImmunoResearch Laboratories. The MF20 anti-sarcomeric myosin heavy chain (MHC) and F5D anti-myogenin antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by The University of Iowa, Department of Biological Sciences. PLD1 antibody was generated by Proteintech Group (Chicago) using a synthetic peptide corresponding to the C-terminal sequences of human and mouse PLD1. IGF2 and gelatin were obtained from Sigma-Aldrich, rapamycin from Calbiochem and all other cell culture reagents were from Invitrogen.

Cell culture

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/l glucose with 10% fetal bovine serum at 37°C with 7.5 % CO_2. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin and grown to 100% confluence, changed into differentiation medium (DMEM containing 2% horse serum), and replenished with fresh medium daily for 3 to 4 days. Transfections were performed using FuGENE-6 (Roche Molecular Biochemicals), and stable pools were obtained by selection in 1 mg/ml G418.

Determination of PLD activity

PLD activity was determined as previously described by measuring [^{3}H]phosphatidylbutanol (PBt) produced in [^{3}H]palmitate-labeled cells via PLD-catalyzed transphosphatidylation (Fang et al., 2003; Yoon et al., 2006). Briefly, C2C12 cells cultured in six-well plates were metabolically labeled with 1 μCi/ml [^{3}H]palmitate for 24 hours. Then the cells were pretreated with 0.3% (vol:vol) 1-butanol for 30 minutes, followed by a brief wash with ice-cold phosphate-buffered saline (PBS) and suspension in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959), and [^{3}H]PBt was separated from other phospholipids by thin layer chromatography on silica G-60 plates, using a solvent system of ethyl acetate:isooctane:acetic acid:water (11:5:2:10, vol:vol). The regions corresponding to the authentic PBt bands were identified with iodine staining, scraped and counted using a scintillation counter.

Western blot analysis

C2C12 cells were scraped in ice-cold PBS and harvested by microcentrifugation. The cells were then resuspended in lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% protease inhibitor mixture), and mixed with SDS sample buffer. Proteins were resolved by SDS-PAGE, transferred onto PVDF membrane (Millipore) and incubated with various antibodies following the manufacturer's recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences).

Immunofluorescence microscopy

C2C12 myoblasts or myotubes grown on tissue culture plates were fixed in 3.7% formaldehyde (in PBS), permeabilized in 0.1% Triton X-100, and then incubated with MF-20 antibody in 3% BSA (in PBS) followed by fluorescein isothiocyanate conjugated anti-mouse IgG in 3% BSA (in PBS), with 4 μg/ml 4,5-diamidino-2-phenylindole (DAPI) whenever applicable. To visualize F-actin, the cells were stained for 20 minutes with Rhodamine-conjugated phalloidin (Invitrogen). The stained cells were examined with a Leica DMI 4000B fluorescence microscope, and the fluorescent images were captured using a RETIGA EXi camera with Qcapture pro51 software (QImaging™), and processed as eight-bit images using Adobe Photoshop CS2. MHC and DAPI signals were pseudo-colored green and red, respectively. The fusion index was calculated from the ratio of nuclei number in myocytes with two or more nuclei versus the total number of nuclei.

Lentivirus-delivered RNAi

All short hairpin (sh)RNAs used in this study were constructed in pLKO.1-puro. shRNAs for mouse *Pld1* and *Pld2* were purchased from Sigma-Aldrich (MISSION® shRNA). Each gene set contained five constructs with distinct target sequences, all of which were packaged for viral production and infection, and tested for target knockdown. Two constructs for each gene with consistent knockdown efficiency were used for further studies. A negative control shRNA (containing a scrambled sequence as target sequence) was obtained from Addgene (Sarbassov et al., 2005). For viral packaging, pLKO-shRNA, pCMV-dR8.91 and pCMV-VSV-G were cotransfected into 293T cells using FuGENE 6 at 0.5 μg, 0.45 μg and 0.05 μg, respectively (in 1 ml for a six-well plate). Media containing viruses were collected 48 hours after transfection. C2C12 cells were infected with the viruses in the presence of 8 μg/ml polybrene (Sigma-Aldrich) for 24 hours, and then subjected to selection by 3 μg/ml puromycin for 72 hours prior to inducing differentiation. Hairpin sequences in these shRNA constructs are as follows: PLD1-1, 5′-CCGGCCCAA-TGATGAAGTACACAATCTCGAGATTGTGTACTTCATCATTGGGTTTTTG-3′; PLD1-2, 5′-CCGGGCTCTCCATGAGAACACGTTACTCGAGTAACGTGTTCT-CATGGAGAGCTTTTTG-3′. (Note: each *Pld1* shRNA targets both isoforms encoded by *Pld1* – PLD1a and PLD1b.) PLD2-1, 5′-CCGGGCAAACAGAAA-TACTTGGAAACTCGAGTTTCCAAGTATTTCTGTTTGCTTTTTG-3′; PLD2-2, 5′-CCGGCATGTCTTTCTATCGCAATTACTCGAGTAATTGCGATAGAAAGAC-ATGTTTTTG-3′. Scrambled, 5′-CCTAAGGTTAAGTCGCCCTCGCTCTAGCGA-GGGCGACTTAACCTTAGG-3′.

Quantitative RT-PCR

Total RNA was extracted from C2C12 cells by using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA with SuperScript II Reverse Transcriptase (Invitrogen) using oligo (dT) primer (Invitrogen). Quantitative (q)PCR was performed on a Bio-Rad iCycler system (Bio-Rad) using a SYBR Green PCR kit (Applied Biosystems) in a MicroAmp 96-well reaction plate (Bio-Rad) following the manufacturer's protocols. β-actin was used as a reference to obtain the relative fold change for target samples using the comparative C_T method. The primers used are as follows: β-actin forward, 5′-TTGCTGACAGGATGCAGAAG-3′; β-actin reverse: 5′-ATCCACATCTGCTG-GAAGGT-3′; IGF2 forward, 5′-CGCTTCAGTTTGTCTGTTCG-3′; IGF2 reverse, 5′-AGGTAGACACGTCCCTCTCG-3′; mPLD1 forward, 5′-AGTGCTTCAGA-CTTGTCCTGGGTT-3′; mPLD1 reverse, 5′-TATGGTAGCGTTTCGAGCT-GCTGT-3′; mPLD2 forward, 5′-TTGCGGAAGCACTGTTTCAGTGTG-3′; mPLD2 reverse, 5′-TTGTTCTCCGCTGTTTCTTGCCAC-3′.

Luciferase reporter assays

C2C12 cells stably expressing H19-luc-ME (Erbay et al., 2003) and infected by various lentiviruses were grown to 100% confluence and induced to differentiate in 2% horse serum. The cells were lysed at the indicated times in Passive Lysis Buffer (Promega), and luciferase assays were performed using the Luciferase Assay Systems kit (Promega) following the manufacturer's protocol.

Measurement of secreted IGF2

IGF2 secreted into C2C12 culture medium was measured using the DuoSet ELISA Development System for mouse IGF2 (R&D Systems) following the manufacturer's protocol.

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