Phosphoinositides generated by phosphoinositide 3-kinase (PI3K) play critical roles in cell metabolism, motility, proliferation, and survival (3). PI3K recruitment to cell membranes in response to extracellular signals leads to the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) that mediates growth factor signaling that leads to cell proliferation, migration, and survival. PI3K-dependent activation of Akt is critical for myoblast differentiation induced by serum withdrawal, suggesting that in these cells PI3K signaling is activated in an unconventional manner. We investigate the mechanisms by which PI3K signaling and Akt are regulated during myogenesis. We report that PI-3,4-P2 and PI-3,4,5-P3 accumulated in the plasma membranes of serum-starved 3T3-L6 myoblasts due to de novo synthesis and increased lipid stability. Surprisingly, only newly synthesized lipids were capable of activating Akt. Knockdown of the lipid phosphatase PTEN moderately increased PI3K lipids but significantly increased Akt phosphorylation and promoted myoblast differentiation. Knockdown of the lipid phosphatase Ship2, on the other hand, dramatically increased the steady-state levels of PI-3,4,5-P3, but did not affect Akt phosphorylation and increased apoptotic cell death. Together, these results reveal the existence of two distinct pools of PI3K lipids in differentiating 3T3-L6 myoblasts: a pool of nascent lipids that is mainly dephosphorylated by Ship2 and is capable of activating Akt and promoting myoblast differentiation and a stable pool that is dephosphorylated by Ship2 and is unable to activate Akt.

Received 30 April 2007/Returned for modification 4 June 2007/Accepted 13 September 2007

Phosphoinositide 3-kinase (PI3K) activation and synthesis of phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) lipids mediate growth factor signaling that leads to cell proliferation, migration, and survival. PI3K-dependent activation of Akt is critical for myoblast differentiation induced by serum withdrawal, suggesting that in these cells PI3K signaling is activated in an unconventional manner. Here we investigate the mechanisms by which PI3K signaling and Akt are regulated during myogenesis. We report that PI-3,4-P2 and PI-3,4,5-P3 accumulated in the plasma membranes of serum-starved 3T3-L6 myoblasts due to de novo synthesis and increased lipid stability. Surprisingly, only newly synthesized lipids were capable of activating Akt. Knockdown of the lipid phosphatase PTEN moderately increased PI3K lipids but significantly increased Akt phosphorylation and promoted myoblast differentiation.

Knockdown of the lipid phosphatase Ship2, on the other hand, dramatically increased the steady-state levels of PI-3,4,5-P3, but did not affect Akt phosphorylation and increased apoptotic cell death. Together, these results reveal the existence of two distinct pools of PI3K lipids in differentiating 3T3-L6 myoblasts: a pool of nascent lipids that is mainly dephosphorylated by Ship2 and is capable of activating Akt and promoting myoblast differentiation and a stable pool that is dephosphorylated by Ship2 and is unable to activate Akt.
contact events, including cell alignment and elongation, which culminate in cell fusion and the formation of multinucleated myotubes. In myoblasts, serum withdrawal promotes Akt phosphorylation and activation in a PI3K-dependent manner (8, 9). Expression of a constitutively active form of PI3K enhances myotube differentiation, while expression of dominant-negative forms of PI3K or treatment with PI3K inhibitors impairs the process (13, 15, 16). Akt activation is thought to mediate the PI3K effects on myogenesis. Constitutively active Akt enhances myotube formation and overcomes the effect of PI3K inhibition, while dominant-negative forms of Akt inhibit the process (12, 32). Despite all the evidence suggesting a role for PI3K in myogenesis, the mechanism by which PI3K signaling is regulated in differentiating myoblasts is not well understood and is of special interest, given that the process is triggered by serum withdrawal and cell cycle arrest.

Here we investigate how phosphoinositide metabolism and Akt activation are regulated during differentiation of 3T3-L6 myoblasts. Our results show that PI-3,4,5-P3 levels are increased during 3T3-L6 myoblast differentiation, reaching maximum levels when the cultures were near confluence and the cells were starting to elongate. Serum withdrawal further and dramatically increased the levels of these lipids due to increased lipid stability. Despite the high levels of PI-3,4,5-P3 and PI-3,4-P2 that accumulate in serum-starved myoblast cultures, Akt phosphorylation was dependent on de novo synthesis of PI3K lipids. Ship2 knockdown in these cells dramatically increased PI-3,4,5-P3 and moderately increased PI-3,4-P2, while Akt phosphorylation was not affected or even decreased. PTEN knockdown, on the other hand, significantly increased Akt phosphorylation but only moderately increased PI3K lipids. Green fluorescent protein (GFP)-PH domain studies showed that PI-3,4,5-P3 was mainly located at the plasma membrane of myoblasts and was concentrated in areas of membrane ruffles but was excluded from areas of cell-cell contact. Immunocytochemistry studies showed that Akt was recruited to membrane ruffles located on the edge of cell extensions, where PTEN and PI-3,4,5-P3 colocalize. Increased Akt phosphorylation in PTEN knockdown cells correlated with enhanced myoblast differentiation, while increased steady-state levels of PI-3,4,5-P3 in Ship2 knockdown cells correlated with increased apoptotic cell death.

MATERIALS AND METHODS

**Cell lines.** 3T3-L6 cells (rat myoblasts), HeLa cells, U87 cells (human glioblastoma cells), and 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). 3T3-L6 cells were subcultured before they reached 50% confluence.

**DNA constructs.** pSuper.retro.puro (OligoEngine) and pRS-eIg-hyg (7) vectors were used to express the short hairpin RNAs (shRNAs) for Ship2 and PTEN. The 19-nucleotide target sequences for Ship2 and Ship2 II shRNAs were 5'-G

**PI3K lipids.** Inorganic 32P for 1.5 to 4 h in phosphate-free DMEM or with 10 μCi/ml [3H]-inositol for 24 or 48 h in inositol-free DMEM supplemented with dialyzed FCS (Gibco) and 200 μM l-glutamine. After the cells were labeled, they were treated as indicated and lysed in 1 N HCl. Lipids were extracted in chloroform-methanol (1:1, vol/vol) and deacylated as described previously (28). Phosphoinositides were separated by anion-exchange high-performance liquid chromatography (HPLC) (Beckman), detected by a flow scintillation analyzer (Perkin-Elmer), and quantified using Palorfin (Perkin-Elmer), which was added with 5% milk dissolved in Tris-buffered saline (TBS) plus 1 m Na orthovanadate. The membranes were probed overnight with the following primary antibodies: anti-phospho-T308 Akt and anti-phospho-S473 Akt (Cell Signalling Technology), anti-Ship2 (Santa Cruz), anti-PTEN (Cell Signaling Technology), anti-total Akt (Cell Signalling Technology), antitubulin (BD Biosciences), anti-phospho-Erk (antibody against phosphorylated extracellular signal-regulated kinase) (Cell Sig-nalling Technology), anti-total Erk (Santa Cruz), anti-phospho-S256 FKHR (Cell Signalling Technology), and anti-phospho-S256 FKHR (Cell Signalling Technology). After the membranes were washed, they were incubated for 60 min with the appropriate secondary antibodies conjugated to IR680 (Rockland and Molecular Probes) or IR800 (Rockland). The membranes were washed in TBS-Tween, and bound antibodies were detected and quantified using the Odyssey infrared imaging system (LI-COR).

**Phosphoinositide analysis.** Cells were metabolically labeled with 200 μCi/ml inorganic [32P] for 1 to 4 h in phosphate-free DMEM or with 10 μCi/ml [3H]-inositol for 24 or 48 h in inositol-free DMEM supplemented with dialyzed FCS (Gibco) and 200 μM l-glutamine. After the cells were labeled, they were treated as indicated and lysed in 1 N HCl. Lipids were extracted in chloroform-methanol (1:1, vol/vol) and deacylated as described previously (28). Phosphoinositides were separated by anion-exchange high-performance liquid chromatography (HPLC) (Beckman), detected by a flow scintillation analyzer (Perkin-Elmer), and quantified using Palorfin (Perkin-Elmer), which was added with 5% milk dissolved in TBS (plus 1 m Na orthovanadate). The membranes were probed overnight with the following primary antibodies: anti-phospho-T308 Akt and anti-phospho-S473 Akt (Cell Signalling Technology), anti-Ship2 (Santa Cruz), anti-PTEN (Cell Signalling Technology), anti-total Akt (Cell Signalling Technology), antitubulin (BD Biosciences), anti-phospho-Erk (antibody against phosphorylated extracellular signal-regulated kinase) (Cell Sig-nalling Technology), anti-total Erk (Santa Cruz), anti-phospho-S256 FKHR (Cell Signalling Technology), and anti-phospho-S256 FKHR (Cell Signalling Technology). After the membranes were washed, they were incubated for 60 min with the appropriate secondary antibodies conjugated to IR680 (Rockland and Molecular Probes) or IR800 (Rockland). The membranes were washed in TBS-Tween, and bound antibodies were detected and quantified using the Odyssey infrared imaging system (LI-COR).
GFP-PH domain localization and immunocytochemistry. 3T3-L6 cells were plated on coverslips and transiently transfected with vector containing the sequence for GFP-PH domain from Btk (a gift from S. Field) or GFP-PH domain from Akt (a gift from S. Grinstein) using Lipofectamine Plus reagent (Invitrogen). Cells were fixed 3 days after transfection with 4% paraformaldehyde in PBS for 10 min, permeabilized, and blocked with 0.3% Triton X-100 in PBS containing 5% donkey serum. For the immunocytochemistry studies, fixed cells (transfected or not) were incubated with primary antibodies overnight, washed, and incubated with the appropriate secondary antibodies conjugated to Cy2 or Cy3 (Jackson Laboratory). Coverslips were mounted in Fluorsave (Calbiochem), and cells were analyzed by confocal microscopy using a Nikon microscope attached to a Bio-Rad confocal microscope.

Apoptosis assay and myoblast differentiation assay. The rate of apoptosis was measured by Western blotting using antibody directed against cleaved caspase 3 (Cell Signaling Technology) as described above, except that semiconfluent cell cultures were scraped off the culture dishes without removing the medium and centrifuged before lysis.

The rate of myoblast differentiation was measured by Western blotting using anti-MHC (anti-myosin heavy chain) antibody (MF20 or F59) as described above or by determining the percentage of fusing colonies in the population. Approximately 500 cells were seeded into a 100-mm tissue culture dish and kept in DMEM supplemented with 10% FCS for 6 days and then in DMEM supplemented with 2% FCS for 2 days. The cells were fixed in 4% paraformaldehyde, and the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) (Calbiochem). The colonies with myotubes containing 20 or more nuclei per cell were counted using a fluorescence microscope.

RESULTS

PI-3,4-P₂ and PI-3,4,5-P₃ levels are induced during 3T3-L6 cell differentiation due to de novo synthesis and increased lipid stability. Myoblasts can be differentiated into myocytes by serum starvation and/or by high cell density. In 3T3-L6 cells, the expression of some myoblast determination markers (such as Myf-5 and MyoD) decreases as the cells start to differentiate. Other differentiation markers (such as myogenin and m-cadherin) are transiently induced just prior to the beginning of the process, while myocyte protein markers, such as MHC, are massively expressed as myotubes start to form. In order to study phosphoinositide metabolism during myogenesis, we metabolically labeled 3T3-L6 cells with [³²P]inositol in regular medium (10% FCS) or in low-serum medium (1% FCS) on different days throughout the process of myoblast differentiation and measured the levels of °H-labeled PI3K lipids. In order to monitor the differentiation process, we also collected protein lysates from cultures maintained under similar conditions and analyzed them for the expression of myoblast differentiation markers (Fig. 1A). Figure 1B shows that PI-3,4-P₂ and PI-3,4,5-P₃ levels significantly increased as 3T3-L6 myoblasts reached confluence (day 4) and were starting to elongate (Fig. 1C to H). The PI-3,4-P₂ and PI-3,4,5-P₃ peaks followed the myogenin peak (not shown) and the m-cadherin peak and preceded the MHC peak (Fig. 1A). These high levels of PI-3,4-P₂ and PI-3,4,5-P₃ were further and dramatically increased (approximately 10-fold) in cells cultured for 24 h in 1% FCS. PI-3-P levels, on the other hand, did not increase with serum withdrawal and decreased only slightly from day 2 to day 3 of the process. Compared to other well-studied cell lines, we found that the maximum levels of PI-3,4-P₂ and PI-3,4,5-P₃ together in 3T3-L6 cells (day 4) were more than 10-fold higher than the levels of these lipids in serum-starved U87 cells (a glioblastoma cell line where PTEN is not expressed) or growth factor-stimulated HeLa cells, labeled under the same conditions (data not shown).

Using °P labeling for short periods, we examined whether PI-3,4-P₂ and PI-3,4,5-P₃ are de novo synthesized after serum withdrawal. For this purpose, 3T3-L6 cells, maintained in regular medium or in low-serum medium for 24 h, were labeled with inorganic °P for 1 to 4 h, and the levels of °P-labeled phosphoinositides were determined. Table 1 shows that °P-labeled PI-3,4-P₂ and PI-3,4,5-P₃ was present in confluent 3T3-L6 cells kept in either 1% FCS or in 10% FCS (equivalent to day 4 [Fig. 1D and G]), demonstrating that this lipid is being synthesized even after 24 h of serum starvation. In contrast, no °P-labeled PI-3,4,5-P₃ peak was detected in sparse 3T3-L6 cultures in either 10% or 1% FCS (equivalent to day 2 [Fig. 1C and F]) even after 4 h of labeling (data not shown), indicating that PI3K activity increases as 3T3-L6 cells become confluent. After 1.5 h of labeling, the levels of °PPI-3,4,5-P₃ in cells growing in 10% FCS were twice as high as in serum-starved cells, while after 3 h of labeling, the levels of °PPI-3,4,5-P₃ in serum-starved cells surpassed the levels in cells growing in regular

| Table 1. Phosphoinositide levels in 3T3-L6 myoblasts labeled with °P or [³²H]inositol
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum concn in cell medium</td>
<td>Lipid</td>
<td>°P</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1.5 h</td>
<td>3 h</td>
<td>4 h</td>
</tr>
<tr>
<td>0.1–1% FCS</td>
<td>PI-3-P</td>
<td>1.004</td>
</tr>
<tr>
<td>PI-3,4-P₂</td>
<td>0.000</td>
<td>0.091</td>
</tr>
<tr>
<td>PI-3,4,5-P₃</td>
<td>0.067</td>
<td>0.093</td>
</tr>
<tr>
<td>10% FCS</td>
<td>PI-3-P</td>
<td>1.099</td>
</tr>
<tr>
<td>PI-3,4-P₂</td>
<td>0.000</td>
<td>0.019</td>
</tr>
<tr>
<td>PI-3,4,5-P₃</td>
<td>0.116</td>
<td>0.083</td>
</tr>
</tbody>
</table>

* 3T3-L6 myoblasts were labeled with °P or [³²H]inositol for the time indicated in medium supplemented with a low or high concentration of serum, as indicated. Data represent the counts detected in each peak as a percentage of the counts detected in the PI-4,5-P₂ peak.

* Data are shown as means ± standard errors of the means.

FIG. 1. Changes in PI-3,4-P₂ and PI-3,4,5-P₃ levels during 3T3-L6 myoblast differentiation. Cells were seeded at a low density (10% confluence) and maintained in medium supplemented with 10% FCS. Twenty-four hours prior to harvesting the lipids or proteins, the medium was changed to DMEM containing 10% FCS (dashed lines) or 1% FCS (solid black lines), supplemented with [³²H]inositol (B, I, and J). (A) The expression of the myogenic markers m-cadherin and MHC during the course of 3T3-L6 differentiation was determined by Western blot analysis. (B) The levels of PI-3,4-P₂ (squares) and PI-3,4,5-P₃ (triangles) in differentiating 3T3-L6 myoblasts were measured by HPLC analysis after metabolic labeling of the cells for 24 h. Also shown are the levels of PI-3-P in cells labeled in medium containing 10% FCS or 1% FCS. (C to H) Phase-contrast microscopy photos of the 3T3-L6 cultures in medium containing 1% FCS (C to E) or 10% FCS (F to H), before cells were collected at day 2 (C and F), day 4 (D and G), or day 5 (E and H). (I and J) HPLC profile of the deacylated phosphoinositides in 3T3-L6 cells labeled for 24 h with [³²H]inositol in medium containing 10% FCS (I) or 1% FCS (J) on day 4 of differentiation. The data shown are representative of more than three independent measurements.
[32P]PI-3,4-P2 was detected only after 3 h of labeling (Table 1), suggesting that it may be a subproduct of PI-3,4,5-P3. Table 1 also shows that the levels of [32P]PI-3-P in serum-starved cells were approximately 10-fold higher than the levels of [32P]PI-3,4,5-P3 and [32P]PI-3,4-P2, which implies that PI-3-P is synthesized at a much faster rate. In contrast, when cells were labeled for 24 to 48 h with [3H]inositol (Table 1), the combined levels of PI-3,4-P2 and PI-3,4,5-P3 were similar to or

FIG. 2. Effects of serum withdrawal and insulin stimulation on the stability of phosphoinositides (PI) and on phospho-Akt levels. (A) 3T3-L6 cells were kept in medium containing 10% FCS (gray symbols) or serum starved in medium containing 1% FCS (black symbols). Twenty-four hours after serum withdrawal, the cells were labeled with [32P]P for 2 h and then treated with wortmannin (100 nM) for 15 or 30 min or not treated, as indicated. The levels of deacylated PI-3-P (circles) and PI-3,4,5-P3 (triangles) were measured after HPLC separation and normalized against total phosphoinositide phosphate levels. After 2 h of [32P] labeling, the levels of [32P]PI-3,4,5-P3 present in serum-starved cells (time zero) were similar to the levels present in non-serum-starved cells. (B) 3T3-L6 cells were labeled with [3H]inositol for 24 h in medium containing 1% FCS and treated with 100 nM wortmannin or not treated for the indicated times, and the levels of deacylated PI-3-P (circles), PI-3,4-P2 (squares), and PI-3,4,5-P3 (triangles) were measured after HPLC separation. Phospho-Akt levels (diamonds) and phospho-Erk1 (stars) in serum-starved cells (1% FCS) treated with 100 nM wortmannin or not treated were measured by Western blotting using anti-pS473 and anti-pErk antibodies. The data shown are representative of more than three independent measurements. (C) 3T3-L6 cells were labeled with [3H]inositol in medium containing 10%, 1%, or 0.1% FCS, and the levels of deacylated PI-3-P, PI-3,4-P2, and PI-3,4,5-P3 were measured after HPLC separation. Phospho-Akt (pAkt) levels were measured by Western blotting, using protein lysates prepared from cells kept under conditions similar to those used for lipid labeling and anti-pS473 antibody. The relative data, normalized against the cells kept in 10% FCS, were plotted in a direct (y axis) and inverse (x axis) logarithmic scale and show the averages ± standard errors (error bars) for three independent experiments. (D) Cells were labeled as described above for panel B, treated with insulin (10 nM) or not treated for 1 min (PI measurements) or 5 min (phospho-Akt measurements), before treatment with wortmannin (white symbols) or DMSO (black symbols) for the time indicated. Time zero indicates the time when wortmannin was added. Time −1 indicates the basal levels of phospho-Akt, and time −3 indicates the basal levels of PI3K lipids. Data were plotted relative to the levels of PIs and phospho-Akt at time zero.
higher than the levels of PI-3-P (see also Fig. 1B, days 3, 4, and 5). Together, these results show that PI-3,4-P₂ and PI-3,4,5-P₃ are synthesized in serum-starved 3T3-L6 cells at a lower rate than in cells growing in regular medium but reach a higher steady-state level, most likely due to increased lipid stability.

To test this possibility, we measured the degradation rates for PI-3-P, PI-3,4-P₂, and PI-3,4,5-P₃ in serum-starved 3T3-L6 cells treated with the PI3K inhibitor wortmannin. First, 3T3-L6 cells (serum starved for 24 h or not) were pretreated with wortmannin or dimethyl sulfoxide (DMSO) for 25 min and then labeled with [³²P] for 1.5 h. In wortmannin-treated cells, PI-3,4,5-P₃ was not detected (data not shown), while in DMSO-treated cells, a [³²P]-labeled PI-3,4,5-P₃ peak was present, demonstrating that the majority of the PI-3,4,5-P₃ synthesized in serum-starved 3T3-L6 cells is sensitive to wortmannin. Next, we measured the degradation rate of newly synthesized PI-3,4,5-P₃ after wortmannin treatment of cells prelabeled with [³²P] for 2 h. Figure 2A shows that PI-3-P levels in serum-starved and non-serum-starved cells decreased at the same rate. In contrast, PI-3,4,5-P₃ levels decreased at a higher rate in non-serum-starved cells than in serum-starved cells (75% and 55% decrease, respectively, after 15 min of wortmannin treatment). After 30 min of wortmannin treatment, 60% of the PI-3,4,5-P₃ in serum-starved cells was wortmannin resistant. These results show that in serum-starved cells, there is a pool of stable PI-3,4,5-P₃, which is 2.5 to 3 times larger than the pool in non-serum-starved cells. To measure the degradation rate of the stable pool of PI-3,4,5-P₃, we labeled cells with [¹¹C]inositol for 24 h prior to wortmannin treatment. Figure 2B shows that, in spite of the dramatic decrease in the steady-state level of PI-3-P, PI-3,4-P₂, and PI-3,4,5-P₃, the level of PI-3-P decreased rapidly (70% decrease after 15 min). In contrast, the steady-state levels of PI-3,4-P₂ and PI-3,4,5-P₃ decreased slowly but steadily. We conclude that serum withdrawal increases the stability of PI-3,4-P₂ and PI-3,4,5-P₃ in differentiating 3T3-L6 cells.

The bulk of PI-3,4-P₂ and PI-3,4,5-P₃ in 3T3-L6 cells cannot stimulate Akt phosphorylation. PI-3,4-P₂ and PI-3,4,5-P₃ were shown to activate Akt by directly binding to its PH domain and to the PH domain of PDK1, bringing these two molecules together to promote the phosphorylation and activation of Akt (3). In order to determine whether the stable pool of PI3K lipids can stimulate Akt phosphorylation, we measured the effects of serum withdrawal on the levels of phospho-Akt and PI3K lipid. Figure 2C shows that, in spite of the dramatic increase in PI-3,4-P₂ and PI-3,4,5-P₃ levels, phospho-Akt levels decreased by at least 50% after serum withdrawal. Erk-1 and Erk-2 were highly phosphorylated in 3T3-L6 cells, and serum withdrawal did not significantly change phospho-Erk levels (not shown). Figure 2D shows that insulin stimulation of serum-starved 3T3-L6 cells leads to small increases in the high basal levels of PI-3,4-P₂ and PI-3,4,5-P₃ (approximately 1.2-fold) and to significant increases in phospho-Akt (9-fold for phosphorylated Ser473 [pS473] and phosphorylated Thr308 [pT308]) and phospho-Erk1/2 (approximately 2-fold) (see also Fig. 3). While wortmannin treatment of 3T3-L6 cells for 15 min decreased serum-starved and insulin-induced PI-3,4-P₂ and PI-3,4,5-P₃ levels by only 20% (Fig. 2B and D), it completely inhibited phospho-Akt levels in serum-starved cells (Fig. 2B), exponentially growing cells (see Fig. 9B), and insulin-treated cells (Fig. 2D). These results demonstrate that Akt phosphorylation is stimulated by newly synthesized PI3K lipids, but not by the bulk of the PI3K lipids that accumulate in 3T3-L6 cells. Interestingly, wortmannin inhibition of Erk1 and Erk2 phosphorylation correlated with inhibition of the stable pool of PI3K lipids (Fig. 2B, phospho-Erk1). PTEN dephosphorylates newly synthesized PI3K lipids, while Ship2 dephosphorylates the bulk of PI-3,4,5-P₃ in 3T3-L6 cells. In order to study the roles of Ship2 and PTEN in regulating the levels of PI3K lipids in 3T3-L6 cells, we suppressed the expression of these proteins using RNAi. Retroviruses derived from pSuper vectors containing the shRNA target sequence for Ship2 (pS-Ship2) or PTEN (pS-PTEN) or a control, unrelated sequence (pS-C1) were used to infect 3T3-L6 myoblasts. Figure 3 shows that in the pS-PTEN-infected cells, 95% of the endogenous PTEN protein was suppressed, while in pS-Ship2-infected cells, 70% of the endogenous Ship2 was suppressed compared to the control cells infected with pS-C1. In pS-Ship2-infected cells, approximately 20% of Ship1 expression was also suppressed (Rat Ship1 and Ship2 sequences share homology in 16 out of the 19 nucleotides present in the target sequence). A second Ship2 target sequence (pS-Ship2 II), which does not affect Ship1 expression, also knocked down 70% of Ship2 expression (not shown), pS-Ship2 infection did not affect the expression of PTEN, and pS-PTEN infection did not affect the expression of Ship2. None of the constructs affected the expression of tubulin, total Erk (Fig. 3), or total Akt (not shown). Figure 3 also shows that 3T3-L6 myoblasts have low basal levels of phospho-Akt (as shown in Fig. 2) and high basal levels of phospho-Erk. PTEN knockdown significantly increased the basal and insulin-stimulated levels of phospho-Akt, but not phospho-Erk. On the other hand, Ship2 knockdown with either pS-Ship2 or pS-
Ship2 II did not affect basal phospho-Akt levels and slightly decreased insulin-stimulated phospho-Akt (discussed below).

We then metabolically labeled the pSuper-infected 3T3-L6 cells before (Fig. 4A) or after (Fig. 4B) differentiation with [3H]inositol to measure the effects of Ship2 and PTEN knockdown on the steady-state levels of PI3K lipids. We also labeled these cells with inorganic 32P to measure newly synthesized lipids before and after insulin treatment (Fig. 5A and B). Ship2 knockdown with pS-Ship2 caused a remarkable increase in the level of PI-3,4,5-P3 and a small increase in the level of PI-3,4-P2 (Fig. 4 and Fig. 5A and B; see also Fig. 8 [discussed below]) but did not significantly affect the level of PI-3-P, PI-4-P, or PI-4,5-P2 (not shown). Ship2 knockdown with pS-Ship2 II also significantly increased PI-3,4,5-P3 levels, but to a lesser extent than with pS-Ship2 (not shown), suggesting that Ship1 may partially compensate for Ship2 knockdown. Increases in PI-3,4,5-P3 levels caused by Ship2 knockdown were observed in more than 20 independent sets of experiments, and the increases ranged from 2- to 20-fold. Ship2 knockdown increased PI-3,4,5-P3 levels regardless of whether the cells were undifferentiated (Fig. 4A and 5) or fully differentiated (Fig. 4B) and regardless of whether the cells were labeled in medium with 10% FCS (not shown) or 0.1 to 2% FCS. After Ship2 knockdown, the levels of PI-3,4-P2 and PI-3,4,5-P3 were often three- to fourfold higher than the levels of PI-3-P. In contrast to Ship2 knockdown, PTEN knockdown caused only a small increase (twofold) to no change in the level of PI-3,4,5-P3 and a modest (two- to threefold) increase in the level of PI-3,4-P2 (Fig. 4 and Fig. 5A and B; see also Fig. 8 [discussed below]). In some labeling experiments, but not all, we also noticed that PTEN knockdown caused small increases (less than twofold) in PI-3-P levels. As with Ship2 knockdown, PTEN knockdown did not significantly affect the level of PI-4-P or PI-4,5-P2.

Together, these results show that Ship2 is an active phosphatase that dephosphorylates PI-3,4,5-P3 in 3T3-L6 cells. In contrast, PTEN seems to have little role in regulating the bulk

![Graph A](http://example.com/grapha.png)

**FIG. 4.** Effects of Ship2 or PTEN knockdown on the steady-state level of phosphoinositide in 3T3-L6 myoblasts and myocytes. Phosphoinositide levels in 3T3-L6 cells infected with pS-C1, pS-Ship2, or pS-PTEN retrovirus were measured in myoblasts (A) or myocytes (B) labeled with [3H]inositol for 48 h in 0.1% or 2% FCS, respectively. The data in panel A represent the averages ± standard errors (error bars) of 5 (control), 10 (Ship2), and 4 (PTEN) independent measurements. The data in panel B represent the average (bars) and range (error bars) for the values from two separate experiments.
Unexpectedly, Ship2 knockdown with pS-Ship2, but not with pS-Ship2 II, also consistently increased the levels of PI-3,4,5-P3 in these cells. Given that Ship2 is a 5′-specific PI-3,4,5-P3 phosphatase, we expected that knocking down Ship2 would cause a decrease, rather than an increase, in the levels of PI-3,4-P2. However, it is possible that the excessively high levels of PI-3,4,5-P3 in pS-Ship2-infected cells caused an increase in the activity of Skip, another PI-3,4,5-P3 phosphatase, that like Ship2 is able to generate PI-3,4-P2. Consistent with this explanation, we found that increased levels of PI-3,4-P2 in Ship2 knockdown cells were detected only after 24 to 48 h [3H]inositol labeling experiments, but not after short 4-h [32P] labeling experiments (compare Fig. 4 with Fig. 5A and B). Alternatively, Ship2 may collaborate with a PI-3,4-P2 phosphatase to coordinate the degradation of both lipids. Therefore, it is likely that the increases in PI-3,4-P2 observed in Ship2 knockdown cells are not a direct consequence of the loss of Ship2 activity.

The effect of Ship2 or PTEN knockdown on basal or insulin-induced phospho-Akt levels was examined (Fig. 3 and Fig. 5C and D). Like many other cells, Akt phosphorylation in serum-starved 3T3-L6 cells is low and is rapidly induced after insulin stimulation (10-fold induction). In Ship2 knockdown cells, despite the robust increase in PI-3,4,5-P3 levels, basal phospho-Akt levels were low and similar to the levels in control cells. After insulin stimulation, phospho-Akt levels increased in Ship2 knockdown cells but to levels slightly (but consistently) lower than the levels in insulin-stimulated control cells. PTEN knockdown, on the other hand, increased basal (3- to 10-fold) and insulin-stimulated (1.5- to 2.25-fold) phospho-Akt levels (see also Fig. 7 and 8 [discussed below]). Similar results were obtained regardless of whether anti-pS473 (Fig. 5C) or anti-pT308 (Fig. 5D) antibodies were used.

The specificity of the effect of Ship2 knockdown on PI-3,4,5-P3 levels was assessed by reexpression of Ship2 protein into Ship2 knockdown cells, as described in Materials and Methods. Exogenous Ship2 expression was twofold higher than
endogenous Ship2, as seen from Fig. 6A (compare the two leftmost lanes). Figure 6B and C show that reexpression of wild-type Ship2 in Ship2 knockdown cells decreased PI-3,4,5-P₃ levels by 60% and increased PI-3,4-P₂ levels by 20%, which is consistent with Ship2 being a 5'/H11032 PI-3,4,5-P₃ phosphatase. Wild-type Ship2 expression had no effect on PI-3,4,5-P₃ levels in control cells expressing endogenous Ship2. Expression of catalytic-dead Ship2, on the other hand, increased PI-3,4,5-P₃ levels in control cells, but not in knockdown cells, consistent with the finding that this mutant can function as a dominant-negative mutant by competing with endogenous Ship2 (33). Expression of wild-type Ship2 did not affect the levels of phospho-Akt in 1% or 10% FCS (not shown). Together, these results confirm that Ship2 is an active PI-3,4,5-P₃-specific phosphatase in 3T3-L6 cells.

FIG. 6. Reexpression of wild-type and catalytic-dead Ship2 in Ship2 knockdown cells. (A) Expression of Ship2 was measured by Western blotting using anti-Ship2 antibody after 3T3-L6 cells infected with pS-C1 or pS-Ship2 were reinfected with empty pBabe virus (−) or pBabe virus carrying the sequence for RNAi-insensitive wild-type (WT) or catalytic-dead (CD) Ship2, as indicated. (B and C) PI-3,4,5-P₃ (B) and PI-3,4-P₂ (C) levels in 3T3-L6 cells expressing RNAi-insensitive wild-type Ship2 (pBabe-Ship2), catalytic-dead Ship2 (pBabe-Ship2-CD), or empty vector (pBabe) were measured by labeling the cells for 48 h with [³H]inositol in medium containing 0.1% FCS. Phosphoinositide levels were plotted relative to the levels in the control cells (cells infected with empty pBabe virus). The results shown are the averages ± standard errors (error bars) obtained from two independent experiments.

FIG. 7. Reexpression of wild-type and lipid phosphatase-dead PTEN in PTEN knockdown cells. Expression of PTEN (A) or Akt phosphorylation (B and C) was measured by Western blotting using anti-PTEN antibody or anti-pS473 and lysates from cells infected with pS-C1 or pS-PTEN reinfected with the pBabe empty vector or pBabe expressing wild-type, RNAi-insensitive PTEN (pBabe-PTEN) or lipid phosphatase-dead PTEN (pBabe-PTEN-LD). The cells in panel B were serum starved for 24 h in medium supplemented with 0.1% FCS, and the cells in panel C were kept in medium supplemented with 10% FCS. Data were normalized against the data for tubulin and are averages ± standard errors (error bars) for three experiments.
The specificity of the effect of PTEN knockdown on phospho-Akt levels was assessed by reexpression of wild-type PTEN or a lipid phosphatase-dead mutant of PTEN, which retains the ability to dephosphorylate peptide substrates (21). The levels of exogenous PTEN expression in 3T3-L6 cells were low and never reached the levels of endogenous PTEN, despite several attempts (Fig. 7A). Nevertheless, reexpression of wild-type PTEN, but not catalytic-dead PTEN (not shown) or lipid-dead PTEN, decreased the basal levels of phosphorylated Akt in PTEN knockdown cells growing in medium containing 10% FCS (Fig. 7C) or 0.1% FCS (Fig. 7B). These results confirmed that lipid phosphatase activity is important for PTEN’s ability to regulate Akt phosphorylation in 3T3-L6 cells, despite the fact that it did not significantly affect the bulk of PI3K lipids in these cells.

Together, these results clearly show that in 3T3-L6 cells the bulk of PI-3,4-P2 and/or PI-3,4,5-P3 cannot stimulate Akt phosphorylation, confirming the results shown in Fig. 2. Most importantly, the data show that PTEN knockdown, but not Ship2 knockdown, induces Akt phosphorylation. Since Akt phosphorylation was shown to be regulated by newly synthesized PI3K lipids, we conclude that PTEN dephosphorylates PI-3,4-P2 and PI-3,4,5-P3 at the sites where they are being synthesized, while Ship2 dephosphorylates the bulk of PI-3,4,5-P3 that turns over slowly and is not involved in Akt activation.

Next, we investigated whether Ship2 can cooperate with PTEN to regulate phosphoinositides and Akt phosphorylation. Double knockdowns of Ship2 and PTEN were generated as described in Materials and Methods. Figure 8A shows the expression of Ship2 and PTEN in these cells. Figure 8A and B show that Ship2 knockdown can further increase Akt phosphorylation (more than twofold) in serum-starved 3T3-L6 cells lacking PTEN expression, but not in cells where PTEN is expressed. Figure 8C and D show the levels of [3H]inositol-labeled PI-3,4-P2 and PI-3,4,5-P3 in serum-starved 3T3-L6. Ship2 and PTEN double-knockdown cells had higher levels of both PI-3,4-P2 and PI-3,4,5-P3 than single-knockdown cells did. In fact, knockdown of Ship2 and PTEN synergized to increase PI-3,4,5-P3 and PI-3,4-P2 and also to increase phospho-Akt levels. Increases in phospho-Akt levels were accompanied by comparable increases in the phosphorylation of the Akt substrates FOXO1 (FKHR), FOXO4 (AFX), and glycogen synthase kinase 3β (data not shown), confirming that measurements of Akt phosphorylation reflected the state of Akt activation in these cells. The finding that Ship2 knockdown regulates Akt when PTEN is absent suggests that Ship2 also dephosphorylates newly synthesized PI-3,4,5-P3 in 3T3-L6 cells. However, PTEN seems to compensate for the loss of Ship2 at the sites of Akt activation.

In non-serum-starved cells, the increases in PI-3,4-P2 and PI-3,4,5-P3 levels caused by Ship2 and PTEN double knockdown were comparable to the increases caused by serum withdrawal of control cells (Fig. 8E and F). These results indicate that in 10% FCS, PI-3,4-P2 and PI-3,4,5-P3 levels are kept low due to the action of these phosphatases. However, serum withdrawal further increased the levels of PI3K lipids in double-
knockdown cells, showing that inactivation of PTEN and Ship2 can only partially explain the increase in lipid stability caused by serum withdrawal.

**Steady-state levels of PI-3,4,5-P3 and PI-3,4-P2 cannot stimulate Akt phosphorylation: possible models.** The observations that serum-starved 3T3-L6 cells have low phospho-Akt levels, despite the high levels of PI-3,4-P2 and PI-3,4,5-P3, and that Ship2 knockdown dramatically increased PI-3,4,5-P3 levels without affecting phospho-Akt, led us to seek potential explanations for this discrepancy. First, we investigated whether PDK2 activity is limiting Akt phosphorylation in serum-starved 3T3-L6 cells, when PI-3,4,5-P3 and PI-3,4-P2 levels are high. Although phosphorylation at S473 is a good indicator of PDK2 activity, it is unclear how phospho-T308 can affect phospho-S473 and vice versa. Thus, we used a constitutively active form of Akt (myr-Akt) to measure phosphorylation at S473, independently of T308. We found that phosphorylation of myr-Akt at S473 was constitutively elevated, as previously observed for other cell lines (36), and was wortmannin insensitive, validating the use of this reagent as a tool for measuring PDK2 activity, independently of T308 phosphorylation. Rapamycin treatment decreased myr-Akt phosphorylation at S473 by 80%, indicating that TORC2 activity accounts for most of the PDK2 activity in these cells. Figure 9A also shows that the levels of PDK2 activity in 3T3-L6 cells with Ship2 and PTEN knockdown were similar to those in control 3T3-L6 cells. The phosphorylation of myr-Akt was high, regardless of whether the cells were cultured in 10% FCS or 1% FCS or treated with insulin (not shown). This result strongly suggests that PDK2 activity is not limiting Akt phosphorylation in 3T3-L6 cells.

In order to investigate whether the low levels of phospho-Akt in Ship2 knockdown cells could be caused by increased dephosphorylation of Akt, we followed the rate of phospho-Akt dephosphorylation after wortmannin treatment. Figure 9B shows that the rate of Akt dephosphorylation was the same, regardless of whether PTEN or Ship2 had been knocked down.

Next, we investigated the possibility that in 3T3-L6 cells the bulk of PI-3,4-P2 and PI-3,4,5-P3 and Akt localize into distinct subcellular compartments. For this purpose, we expressed exogenous GFP-PH domains from Akt (Fig. 10b) or from Btk (Fig. 10a and c to g) in 3T3-L6 cells (Fig. 10a, b, and h to l), in Ship2 knockdown cells (Fig. 10c, d, e, and f) or in Ship2 and PTEN double-knockdown cells (Fig. 10g) and immunostained them for total endogenous Akt, phospho-Akt, PTEN, or m-cadherin. In serum-starved 3T3-L6 cells, the PH domains from Btk and Akt were mostly cytoplasmic, but subfractions clearly localized to specific areas of the plasma membrane (Fig. 10a and b). In serum-starved Ship2 knockout cells, where steady-state levels of PI-3,4,5-P3 are elevated, the GFP-Btk PH domain and endogenous Akt colocalized into spread areas of the plasma membrane or into multiple membrane speckles (Fig. 10c, d, e, and f). We were able to distinguish three distinct patterns of plasma membrane staining by PH domains: (i) the tip of cell extensions, which are rich in actin and found in elongating cells (Fig. 10a, b, d, e, and f); (ii) multiple small membrane speckles, which are common in Ship2 knockout cells (Fig. 10c and g); (iii) membrane ruffles, which are present in serum-starved cells (Fig. 10d, e, and f) and are abundant in insulin-treated cells (not shown). Treatment with wortmannin for short periods (which has been shown in Fig. 2 to inhibit de novo synthesis of PI3K lipids and Akt phosphorylation, but not the steady-state levels of these lipids) decreased the size and abundance of the membrane ruffles. Nevertheless, in wortmannin-treated cells, the PH domains and endogenous Akt clearly colocalized at the cell membrane (Fig. 10f and g). Interestingly, when cells were costained for m-cadherin, we found that the GFP-PH domain of Btk was excluded from regions of cell-cell contact (Fig. 10d).

**Immunostaining of serum-starved 3T3-L6 cells with antibodies against Akt showed that this protein was mostly cytoplasmic but that a subfraction also concentrated at the edge of cell extensions and membrane ruffles and that it was excluded from areas of cell-cell contact** (Fig. 10h). Anti-phospho-Akt anti-
body stained the tip of membrane extensions and membrane ruffles in insulin-stimulated cells or in PTEN knockdown cells only (Fig. 10i and k and not shown). Anti-PTEN antibody also stained the tip of cell extensions (Fig. 10j to l), where Akt and phospho-Akt were also found. In PTEN knockdown cells, PTEN staining at the cell extensions and cytoplasm was significantly decreased (not shown). Together, these results show that in 3T3-L6 cells, PI-3,4-P₂ and PI-3,4,5-P₃ localized to the plasma membrane, where Akt was recruited. In addition, the data also suggest that Akt phosphorylation occurs at specific sites of the membrane, such as membrane extensions, to which PTEN can be recruited.

**Effects of PTEN and Ship2 knockdown on myoblast proliferation, survival, and differentiation.** PI3K signaling has been implicated in several steps of the myogenic process. In order to investigate how the two pools of PI3K lipids affect some of these steps, we measured the effect of Ship2 or PTEN knockdown on proliferation, survival, MHC expression, and fusion. Figure 11A shows a proliferation curve for 3T3-L6 cells infected with pS-C1, pS-Ship2, or pS-PTEN. PTEN knockdown cells can reach a higher saturation density than control cells (twofold increase), but they have similar proliferation rates. In 3T3-L6 cells where Ship2 expression has been suppressed, proliferation rate and saturation density were not significantly affected.

During the process of myogenesis, a large fraction of the myoblasts undergoes apoptosis (34). Confluent Ship2 knockdown cells seem to have increased rates of cell death compared to control cells, as judged by phase-contrast microscopy, especially after serum withdrawal. In the experiment shown in Fig. 11B, we used an antibody against cleaved caspase 3 to measure apoptosis in 3T3-L6 control cells and in Ship2 or PTEN knockdown cells. Confirming our initial observations, we found that Ship2 knockdown with pS-Ship2 caused a dramatic increase in cleaved caspase 3, indicating a correlation between dramatically elevated levels of PI-3,4,5-P₃ and apoptosis.

The effect of Ship2 or PTEN knockdown on myoblast differentiation was determined by measuring MHC levels. Figure 11C shows that PTEN knockdown increased MHC expression in the early stages of fusion, while Ship2 knockdown had no effect. At late stages of myogenesis, there were no significant differences in MHC expression between control 3T3-L6, Ship2 knockdown, or PTEN knockdown (not shown) cells. Figure 11D shows that PTEN knockdown doubled the percentage of colonies in the culture that differentiated into multinucleated myotubes. These results show that PTEN knockdown, but not Ship2 knockdown, can enhance the differentiation process, indicating that Akt activation promotes myoblast differentiation, as suggested by other reports (12). These results also show that
the increases in the steady-state levels of PI-3,4-P₂ and PI-
3,4,5-P₃ by Ship2 knockdown impaired differentiation and en-
hanced apoptosis.

DISCUSSION

The data presented here describe, for the first time, a cell line in which PI-3,4-P₂ and PI-
3,4,5-P₃ accumulate in response to serum withdrawal, as a result of de novo synthesis and
increased stability of these lipids. Matrix-dependent and cell-
cell contact-dependent generation of PI3K lipids has been pre-
viously implicated in the accumulation of PI-3,4,5-P₃ in serum-
starved Cos cells and epithelial cells (17, 35). Therefore, it is
possible that, in confluent myoblasts, synthesis of PI-3,4,5-P₃
after serum withdrawal is due to activation of class I PI3Ks by
extracellular matrix or m-cadherin. Alternatively, PI3K activa-
tion in serum-starved cells may result from increased secretion
of endogenous growth factors (such as insulin-like growth fac-
tor 1, for example) and autocrine stimulation. Our data indi-
cate that in 3T3-L6 cells, PI-3,4-P₂ is generated by dephos-
phorylation of PI-3,4,5-P₃ by Ship2. Although most of the
PI-3,4-P₂ and PI-3,4,5-P₃ present in these cells was wortman-
nin sensitive, we cannot rule out the possibility that class II
PI3K also contributes to PI-3,4-P₂ generation during differen-
tiation.

PI3K activation alone (Table 1) cannot account for the
10-fold increase in PI-3,4-P₂ and PI-3,4,5-P₃ after serum with-
drawal (Fig. 1B). The difference in lipid stability in serum-
starved cells versus non-serum-starved cells (Fig. 2A) is the
most likely cause of the higher steady-state levels of PI-3,4-P₂
and PI-3,4,5-P₃ in differentiating 3T3-L6 cells. The mechanism
for this increased stability is not completely understood, but we
suspect that inactivation of lipid phosphatases may play a role.
In 3T3-L6 cells, PTEN knockdown caused moderate or no
increase in PI3K lipids, indicating that its activity may be re-
stricted to certain subcellular compartments. PTEN activity
was previously shown to be regulated by membrane recruit-
ment (through binding to PDZ-containing proteins) or by
phosphorylation and/or oxidation (19). We speculate that recruitment of PTEN to specific locations of the plasma membrane where PI-3,4,5-P₃ is being synthesized can lead to local activation of this enzyme as a result of a reducing microenvironment.

Unlike PTEN, Ship2 seems to be very active in 3T3-L6 cells. PI-3,4-P₂ is the most abundant PI3K lipid in these cells, and Ship2 knockdown dramatically increased the level of PI-3,4,5-P₃. Surprisingly, Ship2 knockdown alone did not increase Akt phosphorylation, but PTEN and Ship2 double knockdown cooperated to increase PI-3,4,5-P₃ and to induce Akt phosphorylation. The effect of Ship2 knockdown or knock-out on phospho-Akt has been tested in various cell lines. Ship2 was shown to affect growth factor-stimulated Akt phosphorylation in mouse embryonic fibroblasts, but not in 3T3-L1 cells (2, 30). Our results are consistent with the results from Sharrad and Maitland (29), where Ship2 knockdown was shown to increase Akt phosphorylation only in cells where PTEN is absent.

Wortmannin treatment of 3T3-L6 cells together with phophatase knockdown experiments allowed us to distinguish two separate pools of PI3K lipids in these cells: a pool of newly synthesized lipids that is mainly dephosphorylated by PTEN and a pool of stable lipids that is dephosphorylated by Ship2. While newly synthesized PI3K lipids were clearly necessary for Akt phosphorylation, the bulk of PI-3,4-P₂ and PI-3,4,5-P₃ was unable to stimulate Akt phosphorylation. These results are in agreement with the results of Scheid and collaborators, who showed that PI-3,4,5-P₃ was not sufficient for Akt phosphorylation at S473 in mast cells and PI-3,4,5-P₃ was required for full activation of Akt (27). However, in 3T3-L6 cells, we find that PI-3,4-P₂ was not sufficient either, since Ship2 knockdown increased the steady-state levels of PI-3,4-P₂ to the same extent as PTEN knockdown, without increasing phospho-Akt. On the basis of our data, we propose a model where newly synthesized PI3K lipids can diffuse to areas of the membranes where PTEN is absent or inactive to generate the stable pool of lipids. It is possible that the nascent pool of lipids can effectively promote the encounter of PDK1 and Akt, while the stable, but diffuse pool, cannot. Another possibility is that serum withdrawal increases the expression of a PI-3,4-P₂ and/or PI-3,4,5-P₃-binding protein that protects these lipids from being dephosphorylated and prevents them from activating Akt. These findings may help us design novel strategies for regulating Akt activation by a specific stimulus by bringing lipid phosphatases closer to the site of lipid synthesis.

Increased phosphorylation of Akt in PTEN knockdown correlated with enhanced myoblast differentiation, suggesting that the pool of newly synthesized PI3K lipids play an important role in myoblast differentiation through Akt phosphorylation. Although we have been unable to detect elevated levels of PI-3,4,5-P₃ in differentiating C2 myoblasts, we and others have observed that, as for 3T3-L6 myoblasts, Akt phosphorylation increases dramatically in C2 cells as they exit the cell cycle and start to differentiate (9; D. Sarkes et al., unpublished data). Therefore, it is likely that serum withdrawal-induced synthesis of PI3K lipids is a trait of differentiating myoblasts, but only in 3T3-L6 myoblasts, due to the stability of these lipids, this phenomenon detectable.

The role of the stable pool of PI-3,4-P₂ and PI-3,4,5-P₃ in signaling and myoblast differentiation remains to be discovered. We observed that in serum-starved 3T3-L6 cells, phospho-Erk levels were high and were slowly inhibited by wortmannin treatment, as were the bulk levels of the PI3K lipids. Integrin-stimulated Raf-MAPK (mitogen-activated protein kinase)-Erk pathway activation was shown to be PI3K dependent (17). Therefore, it is possible that the stable pool of PI3K lipids can regulate the Raf-MAPK pathway, which has been previously shown to inhibit myoblast differentiation (5, 9) and may be involved in the maintenance of a nondifferentiated population of myoblasts in adult muscle. This possibility is currently being investigated in our lab.

ACKNOWLEDGMENTS

We thank Sergio Grinstein, Alex Toker, Seth Field, and Scott Frank for DNA constructs; Jennifer Chen and Janice Dominov for antibodies; Sheila Thomas for retrovirus reagents; and Lew Cantley and Steen Hansen for insightful discussions.

This work was supported by grant NIDDK 63219 from the National Institute of Health.

REFERENCES


