Cells must increase their mass in coordination with cell cycle progression to ensure that their size and macromolecular composition remain constant for any given proliferation rate. To this end, growth factors activate early signaling cascades that simultaneously promote cell mass increase and induce cell cycle entry. Nonetheless, the mechanism that controls the concerted regulation of cell growth and cell cycle entry in mammals remains unknown. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B pathway regulates cell cycle entry by inactivating forkhead transcription factors and promoting cyclin D synthesis. PI3K/protein kinase B-derived signals also affect activation of p70 S6 kinase and the mammalian target of rapamycin, enzymes involved in cell growth control. We previously showed that enhancement of PI3K activation accelerates cell cycle entry, whereas reduction of PI3K activation retarded this process. Here we examined whether expression of different PI3K mutants affects cell growth during cell division. We show that diminishing or enhancing the magnitude of PI3K activation in a transient manner reduces or increases, respectively, the protein synthesis rate. Alteration of cell growth and cell cycle entry by PI3K forms appears to be concerted, because it results in lengthening or shortening of cell division time without altering cell size. In support of a central role for PI3K in growth control, expression of a deregulated, constitutive active PI3K mutant affects p70 S6 kinase and mammalian target of rapamycin activities and increases cell size. Together, the results show that transient PI3K activation regulates cell growth and cell cycle in a coordinated manner, which in turn controls cell division time.
PI3K Controls Cell Division Time

NIH 3T3 cells, when maintained in exponential growth, show a cell cycle progression rate, because p65PI3K expression shortened division time without altering cell size. Accordingly, expression of the recombinant p85α regulatory subunit, which reduces the magnitude of transient PI3K activation, increased cell division time without altering cell size. These observations illustrate the concerted regulation of cell growth and cell cycle progression rates by PI3K, thereby controlling cell division time. The key role of PI3K in growth control is supported by the observation that expression of a deregulated, constitutive active PI3K form altered p70 S6K and mTOR activation kinetics, giving rise to larger cells.

EXPERIMENTAL PROCEDURES

DNA Constructs, Antibodies, and Materials—pDNA3-TSC1 and pDNA3-TSC2 cDNA and anti-TSC2 antibodies were kindly provided by George Thomas (55). Anti-p70 S6K antibodies were from Santa Cruz Biotechnology, and anti-Thr(P)389 and anti-Thr(P)421/Ser(P)424 p70 S6K antibodies were from New England Biolabs. Horseradish peroxidase-conjugated antibodies were from Dako, and the enhanced chemiluminescence developing kit was from Amersham Biosciences. Rapamycin was from Calbiochem.

Culture and Transfection—NIH 3T3 cells were cultured (37 °C, 10% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) with 10% calf serum (Invitrogen). Stable NIH 3T3 cell lines expressing p110ΔCAAX, p65PI3K and p85α have been described (56, 57). Cell lines expressing D3Ep70S6K were obtained by transfection of cells with Prkç-Myc-D3Ep70S6K cDNA combined with p-Pur cDNA (Clontech); clones were selected in medium containing 2 μg/ml puromycin (Sigma). Transient transfection was performed using LipofectAMINE Plus (Invitrogen) according to manufacturer’s instructions. Cell cycle arrest was as described (27). Briefly, for G₀ phase arrest, cells were incubated without serum for 20 h. For G₁ phase arrest, cells were incubated (20 h) with 5 μM etoposide (Sigma), which yielded 40–50% cells in G₂. For M phase arrest, cells were incubated (20 h) with 0.1 μg/ml colcemid (Invitrogen), yielding ~70% cells in M phase. For G₀ samples, cells were arrested in G₀ for 19 h and incubated with serum for 1 h.

Extract Preparation and Western Blotting—Cells were lysed in 50 mM HEPES pH 8, 150 mM NaCl and 1% Triton X-100 containing phosphatase and protease inhibitors (27, 58). For p70S6K immunoblotting, cells were lysed in 10 mM Heps pH 7.8, 20 mM β-glycerophosphate, 15 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.2% Nonidet P-40 containing phosphatase and protease inhibitors (58). Proteins were separated by SDS-PAGE and equal protein amounts were resolved in SDS-PAGE. Gels were transferred to nitrocellulose and probed with the indicated antibodies.

Cell Labeling—Cells were washed in methionine/cysteine-free RPMI (BioWhittaker) and incubated in this medium supplemented with 10 mM diacyl fetal calf serum for 2 h prior addition of [35S]Met/Cys (20 μCi; Amersham Biosciences) for the times indicated. For 35S Met/Cys labeling, cells were incubated in 10 mM Heps pH 7.8, 20 mM β-glycerophosphate, 15 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.2% Nonidet P-40 containing phosphatase and protease inhibitors (58). Protein concentration was estimated by the BCA assay (Pierce) and equal protein amounts were resolved in SDS-PAGE. Gels were transferred to nitrocellulose and probed with the indicated antibodies.

Cell Size Determinations—To examine cell size after transient transfection and sorting, cells were seeded in 60 mm dishes (2.5 × 10⁵ cells/dish), transfected the following day at 80% confluence using 0.5 μg pEGFP C1 (Clontech) plus 2 μg of plasmids encoding p110ΔCAAX or p70S6K (58), and incubated overnight. The cells were replated in 10-mm dishes, incubated alone or in the presence of rapamycin (20 nM, 72 h), harvested, and sorted for GFP expression. Forward scatter profiles were analyzed by live cell flow cytometry using a Becton Dickinson fluorescence-activated cell sorter.

To determine cell size in stable transfected cell lines, the cells were maintained in exponential growth, alone or in the presence of rapamycin (20 nM, 4 days). The cell diameters and volumes were determined using a particle size counter (CASY, Särchen System).
RESULTS

PI3K Deregulation Alters Cell Growth—We previously examined the consequences on cell cycle progression of interfering with physiological PI3K activation kinetics by expressing different PI3K forms (27). These studies indicated that enhanced PI3K activation accelerates cell cycle entry, whereas decreased PI3K activation reduces this transition, supporting the role of PI3K in cell cycle entry. PI3K activation must nonetheless be transient to allow completion of the cell cycle, because expression of a constitutive active PI3K or PKB mutant deregulated forkhead transcription factor activity throughout the cell cycle, impairing mitotic progression (27).

Here we analyze whether PI3K regulates cell growth in exponentially dividing mammalian cells. We examined growth in NIH 3T3 cell lines expressing p110<sub>caax</sub>, a constitutively active p110 catalytic subunit mutant (57), or p65<sub>PI3K</sub>, a mutant of the PI3K p85<sub>H9251</sub> regulatory subunit that binds to p110 and enhances its activation by growth factors (56). We also studied cell lines expressing recombinant p85<sub>α</sub> at levels double those of the endogenous protein; this modification reduces the magnitude of endogenous p110 activation (56, 57). The cells were maintained in exponential growth and labeled for short periods with [35S]Met/Cys to compare protein synthesis rates. A 30-min labeling period was adequate to obtain sufficient labeling without saturation; Fig. 1 illustrates a representative experiment and quantification of several assays. Whereas p85<sub>α</sub> expression reduced [35S]Met/Cys incorporation, the two activating PI3K mutations, p110<sub>caax</sub> and p65<sub>PI3K</sub>, increased the protein synthesis rate.

We also compared protein synthesis rates in these cell lines that had been arrested in different phases of the cell cycle. As reported, protein synthesis in control cells was maximal in G<sub>1</sub> (1, 3) and was moderate in G<sub>0</sub>- and G<sub>2</sub>-arrested cells (Fig. 2). Protein synthesis was also low in M phase arrested cells (not shown). G<sub>0</sub>-arrested p65<sub>PI3K</sub>-expressing cells showed a higher rate of protein synthesis than control or p85<sub>α</sub>-expressing cells. This may be due to the modest basal activation of PI3K seen in p65<sub>PI3K</sub> cells (56). Nonetheless, p110<sub>caax</sub>-cells exhibited a remarkably higher level of protein synthesis than control or p85<sub>α</sub>-expressing cells. This may be due to the modest basal activation of PI3K seen in p65<sub>PI3K</sub> cells (56).

FIG. 1. PI3K enhances growth in NIH 3T3 cells. Exponentially growing stable cell clones expressing p65<sup>PI3K</sup>, p85<sub>α</sub>, or p110<sub>caax</sub> were labeled with 20 μCi of [35S]methionine. A, cells were collected after 30 min of labeling, and 20 μg of total lysate was analyzed by SDS-PAGE and autoradiography. B, total cpm at each time point were quantified using phosphorimaging; the graph shows the mean cpm incorporation of five different experiments at 30 min of labeling. Ctr, control.

FIG. 2. Sustained PI3K activation enhances growth throughout the cell cycle. Stable cell clones expressing p65<sup>PI3K</sup>, p85<sub>α</sub>, or p110<sub>caax</sub> were arrested in G<sub>0</sub>, G<sub>1</sub>, or G<sub>2</sub> and were labeled with 20 μCi of [35S]methionine. A, cells were collected after 30 min, and 20 μg of total lysate was analyzed by SDS-PAGE and autoradiography. B, total cpm at each time point were quantified using phosphorimaging; the graph shows the mean cpm incorporation of two experiments. Ctr, control; FS, forward scatter.

FIG. 3. Sustained PI3K activation increases cell size. p65<sup>PI3K</sup>, p85<sub>α</sub>, or p110<sub>caax</sub> clones were cultured in DMEM with 10% calf serum (CS) and then harvested for analysis by live cell flow cytometry. Overlaid forward scatter profiles are shown.
expressing cells nonetheless exhibited higher rates of biosynthesis in all cell cycle phases.

We postulated that PI3K may control cell growth and cell cycle progression rates in a concerted manner, giving rise to cells that are normal in size but that divide more rapidly or more slowly, depending on the intensity of PI3K activation. We measured the size of the stable cell lines expressing the different PI3K forms by flow cytometry. Both p85α- and p65PI3K-expressing cells showed a similar size to that of NIH 3T3 control cells (Fig. 3). Nonetheless, cells expressing the constitutive active p110caax mutant were larger than controls (Fig. 3).

We also analyzed cell diameter and volume using a particle size counter. The means of 10 determinations are shown.

| Cell type      | Mean diameter ± S.D. | Δ diametera | Diameter p valueb | Mean volume ± S.D. | Δ volumeb | Volume p valueb |
|----------------|----------------------|-------------|-------------------|-------------------|-----------|-----------------|
| Control        | 17.13 ± 0.84         | 1.31        | 0.02              | 2936 ± 509        | 353       | 0.18            |
| p65PI3K        | 17.70 ± 1.17         | 0.57        | 0.19              | 3299 ± 694        | 462       | 0.26            |
| p85α           | 17.90 ± 1.28         | 0.77        | 0.15              | 3398 ± 593        | 462       | 0.26            |
| p110caax       | 19.34 ± 0.93         | 2.21        | <0.001            | 4124 ± 461        | 1188      | <0.001          |

a Δ diameter or volume was calculated by subtracting the mean diameter or volume of the control cell line from the mean diameter or volume of each individual cell line.
b Student’s t test. p values were obtained by comparing the raw values for diameter or volume of each stable cell line with those for control cells.

mTOR in turn regulates 4EBP1 and phosphorylated 4EBP1 species in control cells following serum stimulation (G1) (Fig. 5A). This species was nonetheless already found in serum-starved p110caax cells and was more clearly detectable in p110caax cells than in controls in all cell cycle phases (Fig. 5A). This suggests that p110caax expression affects mTOR activation. These results show that deregulation of PI3K affects mTOR and p70 S6K activity.

To analyze whether p70 S6K deregulation was exclusively a consequence of defective mTOR inactivation, we overexpressed TSC1 and TSC2, which inhibit mTOR (30–32). Transfection of the exogenous TSC1/2 complex in p110caax cells reduced 4EBP1 mobility as well as p70 S6K activation levels in G1 (Fig. 5B) but did not correct the prolonged activation kinetics of p70 S6K in G2/M (Fig. 5B). This supports mTOR deregulation as a contributory mechanism to cell mass increase in p110caax cells. In addition, even when mTOR is inhibited by TSC1/2 expression, p110caax induces prolonged p70 S6K activation, reflecting a direct PI3K effect on p70 S6K activation that may also contribute to increasing the size of p110caax cells.
PI3K Controls Cell Division Time

Enhanced Activity of p70 S6K and mTOR Mediates p110caax Cell Size Increase—To examine whether increased p110caax cell size was a consequence of enhanced p70 S6K and mTOR activation, we inhibited these enzymes using rapamycin (33). Control and p110caax-stable transfectants were cultured alone or with rapamycin, and their volumes were measured in a particle size counter (Table I). Rapamycin decreased the volume of control cells moderately (−10%) and that of p110caax cells more intensely (>20%) (Table I). We also measured the volume of stable transfectants of the p70 S6K mutant D3E p70 S6K, an activating mutation with acidic substitutions in the pseudosubstrate region residues (Ser411, Ser418, Thr421, and Ser424) but whose activity requires Thr389 and Thr229 phosphorylation, remaining sensitive to rapamycin (55). As for p110caax cells, the D3E p70 S6K-expressing cells were larger, and their size decreased by ~20% following incubation with rapamycin (Table I).

In an alternative approach, the cells were transiently transfected with a vector encoding GFP and either a control vector or cDNA encoding p110caax. The cells were then incubated alone or in the presence of rapamycin, and GFP-positive and negative cells were isolated by cell sorting (transfection efficiency, ~60%). p110caax-transfected cells were larger than control cells (Fig. 6). Nonetheless, control and p110caax cells were similar in size when incubated with rapamycin (Fig. 6). The cells were also transfected with cDNA encoding p70 S6K, which gave rise to larger cells; this phenotype was also attenuated by rapamycin addition (Fig. 6). Similar results were obtained using the constitutive active p70 S6K mutant D3E p70 S6K (not shown). As the size of p110caax cells decreases upon inhibition of p70 S6K and mTOR, these results indicate that PI3K increases cell growth by affecting mTOR and p70 S6K regulation. Our observations support the hypothesis that transient variations in the magnitude of PI3K activation modify growth and cell cycle progression rates in concert. In contrast, sustained PI3K activation deregulates cell growth machinery throughout the cell cycle, uncoupling the protein synthesis rate from cell cycle progression rates, giving rise to larger cells.

**DISCUSSION**

The observations presented show that alteration of endogenous PI3K activation by expression of PI3K-interfering forms (p65PI3K, p85α, or p110caax) affects the rate of cell growth in dividing mammalian cells. Moreover, expression of p65PI3K or p85α, which induces transient enhancement or reduction in the magnitude of PI3K activation, alters cell growth without significantly modifying cell size (Fig. 3). This shows that transient changes in the intensity of PI3K activation modify cell cycle progression in concert with cell growth rates. In fact, the regulated increase in PI3K activation induced by p65PI3K accelerated cell division (decreased t1/2), whereas the p85α-triggered reduction in the magnitude of PI3K activation delayed cell division (Fig. 4). These observations suggest that cells sense the magnitude of PI3K activation and establish a cell cycle progression rate that is proportional to the cell growth rate, ensuring that daughter cells maintain appropriate cell size. The critical role of PI3K in the control of cell growth during cell division is supported by the observation that expression of a deregulated, constitutive active PI3K mutant impairs coordination of these two processes, inducing a cell size increase. This is the first description that links transient PI3K activation to the concerted regulation of cell growth and cell cycle progression rates during cell division in mammals.

Expression of the constitutive active PI3K mutant p110caax induces enlargement in cell size (Fig. 3). This mutant increases the protein synthesis rate (Fig. 1) and accelerates cell cycle entry but retards G2/M progression and cell cycle exit (27). A partial explanation for the lack of balance between cell growth and cell cycle progression rates in p110caax cells may thus be the delayed transition through G2/M. We nonetheless show
that constitutive activation of PI3K also interferes with correct down-regulation of cell growth-promoting pathways. Accordingly, incubation with PI3K inhibitors reduces cell growth (35) and impairs cell cycle entry (24, 27, 35). We examined mTOR and p70 S6K and found that p110Δcaax expression extended p70 S6K activation kinetics to the G2/M phases and induced hyper-phosphorylation of the mTOR effector 4EBP1 in G1. PI3K activation must thus be transient to allow correct control of cell cycle progression (27) and cell growth throughout the cell cycle.

The reduction in p110Δcaax cell size following rapamycin inhibition of mTOR and p70 S6K activity suggests that these PI3K effectors control growth in dividing cells. PI3K may regulate cell growth by additional mechanisms. This possibility is supported by the behavior of NIH 3T3 p65ΔPI3K-expressing cells, in which p70 S6K is transiently triggered and down-regulated, but whose activation levels are lower than in controls (not shown). This concurs with our previous observations showing that p85Δ, but not p65ΔPI3K, forms a complex with p70 S6K and mTOR that is required for p70 S6K activation (58). Stable NIH 3T3 p65ΔPI3K cells express similar levels of p65ΔPI3K and of endogenous p85 (56), which accounts for the moderate p70 S6K activation observed in these cells. Because p65ΔPI3K cells have a higher protein synthesis rate and reduced p70 S6K activation; p70 S6K does not appear to be the main effector mediating enhanced cell growth in these cells. Activation of the p70 S6K 2 isoform (51, 60), TSC inactivation (30–32), or an as yet undescribed mechanism may cooperate with p70 S6K to enhance cell growth in response to PI3K activation. In addition, it was recently reported that PI3K enhances 5′ TOP mRNA translation independently of p70 S6K (50). We found that p65ΔPI3K-expressing cells have a higher proportion of rpl32 mRNA (5′ TOP) (50) in heavy polysomes than control cells (not shown), suggesting that 5′ TOP translation is enhanced in these cells.

The observations presented suggest that PI3K has an essential role in the concerted regulation of cell growth and cell cycle progression. Previous observations in yeast illustrated that inhibition of cell growth blocks cell cycle entry, whereas inhibition of cell cycle progression allows growth to continue (6). This shows that cell cycle entry is linked to the cell growth process. As to the signaling pathways that control cell growth in yeast, no class I PI3K homologues have been found in this organism; TOR function in control of cell growth is nonetheless conserved from yeast to mammals (61).

In the fruit fly Drosophila melanogaster, disruption of cell cycle regulatory genes (de2F and cdc2) results in cell cycle arrest at a larger cell size (62, 63). This shows that growth without division can also be observed in this organism, but division requires growth. With regard to the pathways that control cell growth and cell division, mutations in Inr, dp110, dIRS (Chico), dPTEN, and dRas affect cell growth and cell cycle simultaneously, whereas mutations in dTOR, d4EBP, and dS6K affect only cell size (reviewed in Refs. 4, 7–10, and 64). In addition, deletion of the negative regulator TSC1 (which participates in negative control of TOR) affects cell size (4, 5, 65). PI3K regulates the TSC complex and TOR (7, 30–32), suggesting that one signaling branch downstream of PI3K regulates cell growth, and the other controls cell cycle progression. dAKT appears to regulate only cell size, suggesting that AKT lies in the growth branch of the PI3K pathway in flies (66). Another difference compared with mammals is that dS6K appears to lie in a pathway different from that of dPI3K (67), although the dPI3K pathway still controls cell growth and cell division. Most of the mutations mentioned above were described in the Drosophila wing imaginal disc, in which cell growth and cell cycle increase in parallel. The study of these processes in Drosophila has the additional difficulty that organ size is subject to internal regulatory mechanisms (reviewed in Refs. 4, 5, 7, and 64). Moreover, division is not coupled to growth in some organs; for example, in the pupal stage, post-mitotic cells in the eye grow without undergoing division (4). This explains the observation that flies carrying a dp110 mutation exhibit a cell growth and cell division phenotype in the wing but only a growth phenotype in the eye (8).

In mammals, inhibition of cell growth also blocks cell cycle entry (3), although growth continues following inhibition of cell cycle entry (35). This also shows that growth in mammals can be separated from the cell cycle but that the cycle is linked to growth. Cell growth in mammals requires PI3K and TOR activities; in fact, expression of the p16 cell cycle inhibitor blocks the cycle in G1, but the resulting cells are larger (35). This cell size increase is partially blocked by TOR inhibition and even more clearly by PI3K inhibitors, illustrating the relevance of PI3K and TOR in cell growth control (35). Nonetheless, only PI3K, but not TOR, appears to mediate the concerted regulation of cell growth and cell cycle (Fig. 4). In contrast to the ability of PI3K mutants to regulate cell cycle progression and growth, activation of the mTOR pathway does not trigger cell division (5, 9, 35). PI3K is thus the first signaling pathway reported to link both processes. Two routes would be induced by PI3K, one branch involved in triggering cell cycle entry and the other in promoting cell growth. The branch regulating cell growth includes mTOR and its effectors, among others (5, 28–32, 50). Regulation of cell cycle entry downstream of PI3K requires Rac, Cdc42, and PKB activation, which affects cyclin/CDK activities or stability (4, 24–27, 62, 68). Nonetheless, PI3K involvement in coordinating cell growth and cell division was not observed in mice expressing constitutive active forms of PI3K/PKB in the heart (52, 53). Expression of constitutive active PI3K/PKB in post-mitotic

**Fig. 6. Incubation with rapamycin reduces p110Δcaax cell size.** NIH 3T3 cells cultured in DMEM with 10% CS were transiently transfected with a PG5 empty vector, a vector encoding p110Δcaax, or a vector encoding p65ΔPI3K, all in combination with a vector encoding GFP (4:1). At 24 h post-transfection, the cells were plated alone or with rapamycin (20 nM) and incubated for 72 h. The cells were harvested and sorted for GFP expression and then analyzed by live cell flow cytometry. Overlaid forward scatter profiles of GFP(+) and GFP(−) cells are shown. Ctr, control.
cells (cardiomyocytes) may mask the contribution of PI3K to triggering cell division (52, 53). In contrast, the phenotype of mice expressing the transgenic p65pi3K mutation as a transgene in T cells and retina revealed the contribution of the PI3K route in cell division in vivo (69, 70).

The mechanism by which PI3K exerts concerted regulation on cell cycle progression and cell growth is incompletely understood. Induction of cell growth and cell cycle entry may simply occur in parallel. Because both cell growth and cell cycle entry are regulated by PI3K, the magnitude of PI3K activation may determine the extent of these processes. It is also possible that translation of a specific cell cycle entry component is sensitive to the availability of the translation machinery. In yeast, G1 cyclin (Cln3) protein expression is highly dependent on the levels of the translation initiation complex, such that Cln3 levels define whether a cell has sufficient translation machinery to enter the cell cycle (71). It has also been shown that overexpression of cyclin D in Drosophila triggers cell growth (4), supporting the possibility that cyclin E rather than cyclin D acts as a growth sensor in this organism. In mammals, PI3K contributes specifically to inducing cyclin D and E synthesis and regulates E2F induction (24, 72). Nonetheless, whether or not translation of mammalian G1 cyclins mRNAs depends on PI3K-controlled translation machinery remains to be determined.

The fact that PI3K has a crucial role linking cell growth and cell cycle entry does not imply that this enzyme is in itself sufficient for either of these processes. For instance, 5' cap translation, which accounts for 85% of total translation, requires mTOR activation. Nonetheless, mTOR activity requires not only TSC inactivation by PI3K/PKB (30) but also appropriate ATP and nutrient levels (40, 41). Translation initiation is also regulated by mitogen-activated protein kinase-dependent pathways (73). For cell cycle entry, other signaling cascades in addition to PI3K also modulate cyclin D expression (74, 75). The requirement for signals other than PI3K to induce cell growth or division explains why some receptors that activate PI3K can induce cell growth, whereas others trigger cell division (4, 5, 50, 72). It is thus possible that the pathways that act in conjunction with PI3K to trigger cell growth and cell cycle entry also have a role in coordinating these two processes. Nonetheless, the concerted modification of cell cycle progression and cell growth rates observed after genetic alteration of PI3K points to this early signal as a central player for correct coordination.

In conclusion, alteration of tsc by without modification of cell size or cell cycle profiles in p65pi3K and p8aO-expressing cells illustrates the central role of PI3K in the concerted regulation of cell growth and cell cycle progression. The upstream position of PI3K in cell growth- and cell cycle-controlling signaling pathways makes this regulation possible. Coordination of both processes requires PI3K activation to be transient.

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