Temporal and Spatial Regulation of Phosphoinositide Signaling Mediates Cytokinesis

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Summary

Polarity is a prominent feature of both chemotaxis and cytokinesis. In chemotaxis, polarity is established by local accumulation of PI(3,4,5)P3 at the cell’s leading edge, achieved through temporal and spatial regulation of PI3 kinases and the tumor suppressor, PTEN. We find that as migrating D. discoideum cells round up to enter cytokinesis, PI(3,4,5)P3 signaling is uniformly suppressed. Then, as the spindle and cell elongate, PI3 kinases and PTEN move to and function at the poles and furrow, respectively. Cell lines lacking both of these enzymatic activities fail to modulate PI(3,4,5)P3 levels, are defective in cytokinesis, and cannot divide in suspension. The cells continue to grow and duplicate their nuclei, generating large multinucleate cells. Furrows that fail to ingress between nuclei are unable to stably accumulate myosin filaments or suppress actin-filled ruffles. We propose that phosphoinositide-linked circuits, similar to those that bring about asymmetry during cell migration, also regulate polarity in cytokinesis.

Introduction

Cytokinesis is the culminating event of the cell cycle that divides a parent cell into two daughter cells. In animals, the dividing cell usually rounds up, then elongates and begins to constrict at the midline. The membranes from opposite sides of the cell invaginate, then fuse, pinching off the newly formed progeny cells. In this dynamic process, cellular division is highly coordinated with mitotic segregation of the chromosomes and distribution of the cellular organelles. If the process is not properly regulated, components can be mislocalized or division can fail completely (Guerin et al., 2002; Robinson and Spudich, 2000; Schweitzer and D’Souza-Schorey, 2004; Wang et al., 2003). Cytokinesis is a potential therapeutic target for drugs, and its inhibition might prevent excessive cell proliferation as occurs in cancer (Miyamoto et al., 2003).

In cytokinesis, a coordinated network of dynamic events takes place at the furrow and poles of the dividing cell. A series of proteins, including myosin II, Rho GTPases, and cortezxillin, associate with the furrow in multiple organisms, while others such as Rac E, dynactin, coronin, and scar localize to the poles (Bi et al., 1998; De Lozanne and Spudich, 1987; Drechsel et al., 1997; Kishi et al., 1993; Kitayama et al., 1997; Lippincott and Li, 1998; Weber et al., 1999). Actin filaments play a key role at the poles as well as at the furrow. Newly polymerized actin and actin binding proteins associate with polar ruffles, while actin and myosin filaments accumulate in the furrow and contribute to the contractile ring that pinches the daughter cells apart (Fukui, 2000; Gerisch et al., 1999; Cao and Wang, 1996; O’Connell et al., 2001). Mutations or mislocalizations of these cytoskeletal proteins cause severe defects in cytokinesis. For example, in Dicyostelium discoideum, deletion of myosin II prevents cytokinesis when cells are grown in suspension. An important aspect of the cytokinesis machinery, however, is its resiliency. Many of these cytoskeletal proteins are individually dispensable for cytokinesis under specific conditions. For instance, when the myosin II null mutants are grown on surfaces, they are able to divide. In addition, D. discoideum cells can undergo “traction-mediated cytofission,” in which multinucleated cells fragment and move apart.

Cells must acquire and maintain distinct cortical asymmetries for many critical cellular functions, including chemotaxis and cytokinesis. Using D. discoideum as a model system, the chemoattractant-induced mechanism that establishes asymmetry of migrating cells has been delineated: Receptor/G protein signaling directs PI3 kinases and PTEN to relocate to regions of the membrane exposed to higher and lower chemoattractant concentration, respectively (Devreotes and Janetopoulos, 2003; Funamoto et al., 2002; Iijima et al., 2002). PI(3,4,5)P3 accumulates locally in the membrane at the front and aligns the cell along the gradient (Parent and Devreotes, 1999). The elevated phosphoinositide levels bias actin filament formation and pseudopodia extension to occur at the front. The decreased levels of PI(3,4,5)P3 at the rear of the cell prevent extraneous projections in this region and help specify the back (Chen et al., 2003). In cells lacking PTEN, PI(3,4,5)P3 and actin polymerization levels are coordinately elevated, the zone of pseudopodia formation is broadened, and the “polarity circuit” is compromised (Iijima and Devreotes, 2002). While the mechanisms of enzyme activation/inhibition have not been established, a similar local accumulation of PI(3,4,5)P3 controls polarity in many cells, including neutrophils and fibroblasts (Haugh et al., 2000; Wang et al., 2002). In fact, loss of polarity and enhanced motility may contribute to the invasive and metastatic properties of mammalian cells lacking PTEN (Goberdhan and Wilson, 2003; Higuchi et al., 2001; Liliental et al., 2000).

While polarity during cytokinesis depends on internal cues rather than an external chemoattractant, the mechanisms for its establishment may be similar. Current thinking holds that during cytokinesis microtubules associated with the mitotic apparatus interact with and control the cell cortex and that the cortex, in turn, may affect the progress and orientation of the spindle (Cao and Wang, 1996; Rappaport, 1986; Rieder et al., 1997; Robinson and Spudich, 1998; Rieder et al., 2004; Rosenblatt et al., 1998; Wheatley and Wang, 1996). Surprisingly, signaling events at the plasma membrane that may regulate the cy-
toskeleton have received relatively little attention even though there have been reports that phosphoinositides and other phospholipids participate in cytokinesis (Brill et al., 2000; Iijima and Devreotes, 2002; Janssen and Schleicher, 2001). It seems natural that signaling events would play an important role in cytokinesis, as they do in cell migration. The bipolar shape of a cell during cytokinesis can be conceptualized as two oppositely polarized cells moving apart from each other, the poles being equivalent to the “fronts” of the two forming daughter cells, and the furrow corresponding to the “backs.” Because of these morphological parallels between cell division and migration, we hypothesized that PI(3,4,5)P₃ might localize at the poles and influence the polarity of the actin cytoskeleton during cytokinesis. In fact, our results show that a “polarity circuit” involving the temporal and spatial regulation of PI(3,4,5)P₃ metabolism plays a key role in cell division.

Results

Signaling Components Function Locally during Cytokinesis

To test the hypothesis that the spatial distribution of PI(3,4,5)P₃ controls polarity during cell division, we examined distributions of a biosensor for this phosphoinositide, PH²⁺Crac-GFP, in *D. discoideum* during cytokinesis. As shown in Figures 1A and 1B and Movie S1 (see the Supplemental Data available with this article online), prior to cytokinesis, PI(3,4,5)P₃ was localized to ruffles at the leading edges of migrating cells. As the cells rounded up at the onset of cytokinesis, PI(3,4,5)P₃ signaling was suppressed, and PH²⁺Crac-GFP, no longer apparent on the membrane, was elevated in the cytosol (second panel in Figure 1B). Then, as cytokinesis progressed, the phosphoinositide concentrated on ruffles appearing at the poles of the dividing cell and was absent from the forming furrow (Figure 1C and Movie S2). Following division, PI(3,4,5)P₃ was again selectively localized on the anterior projections of the two daughter cells. In cells undergoing traction-mediated cytofission, there was a similar bipolar distribution of PI(3,4,5)P₃ on the membrane at the diverging ends of the cell (Figure S1; see the Supplemental Data available with this article online).

We next asked whether these local accumulations of PI(3,4,5)P₃ are controlled by reciprocal regulation of PI3 kinase (PI3K) and PTEN, as occurs in cell migration. In dividing cells, PI3K2-GFP was invariably associated with F-actin-based ruffles occurring at the poles. In contrast, PTEN-GFP was associated with the membrane in the central region of the cell that was devoid of ruffles. These localizations were similar in cells undergoing traction-mediated cytofission (data not shown). The distributions were maintained as the process of division progressed, and, as the cells separated, PI3K2-GFP was sharply localized at the anterior of the two daughters, while PTEN-GFP was clustered at the rear (Figure 1D; Movies S3 and S4). Furthermore, as shown in Figure 1E, when cells rounded up to enter cytokinesis, PTEN-GFP intensely associated with the entire membrane. This distribution is consistent with the suppression of PI(3,4,5)P₃ signaling observed during this phase (Figures 1A and 1B). As the spindle elongated, PTEN dissociated from the poles and concentrated at the middle and along the lateral edges. These movements of PTEN mirror those of myosin II (see Figure S5 below).

Perturbation of the Spatial Distribution of PI(3,4,5)P₃ Inhibits Cytokinesis

To test whether the localized distributions of PI(3,4,5)P₃ played a role in cytokinesis, we examined the growth of existing mutants with altered phosphoinositide levels. As shown in Figure 2A, deletion of PI3K1 and PI3K2, which lower PI(3,4,5)P₃ levels, or deletion of PTEN, which prolongs chemoattractant-induced changes in PI(3,4,5)P₃, each increased the doubling time. Similarly, PI3K inhibitors interfered with cytokinesis (see below). The effects of inactivation of PTEN were stronger (Huang et al., 2003; Iijima and Devreotes, 2002), suggesting that preventing the degradation of PI(3,4,5)P₃ is more deleterious than lowering levels. However, even with inhibitors or in the *pi3k¹⁻²⁻* or *pten⁻* cells, there still is modulation of PI(3,4,5)P₃. This is because each activity is independently regulated in a reciprocal manner (Chen et al., 2003; Ma et al., 2004). In order to further impair regulation, we perturbed both PI3K and PTEN activities. Starting with the cell line carrying deletions in PI3K1 and PI3K2 (*pi3k¹⁻²⁻* cells), we also deleted PTEN, generating a new line lacking all three enzymes (*pi3k¹⁻²⁻pten⁻* cells). The Southern blot in Figure S1 demonstrates that the PTEN locus was disrupted as predicted. As anticipated, the deletion of both activities generated a cell line that lacked temporal and spatial regulation of PI(3,4,5)P₃ (see below).

As shown in Figure 2A, these cells were unable to divide in suspension, and we sought the origin of the defect. As the *pi3k¹⁻²⁻pten⁻* cells were shaken for several days, they became increasingly large. The fold increases in protein mass during overnight culture were essentially the same for the wild-type and *pi3k¹⁻²⁻pten⁻* cells (4.8 and 4.9, respectively). As shown in Figure 2, when examined by Nomarski DIC optics after 3 days, the diameters of the largest cells ranged up to 120 μm, whereas the typical wild-type cell was about 15 μm. Although the *pi3k¹⁻²⁻pten⁻* cells failed to increase in...
number, the nuclei continued to divide. The rate of nuclear division was essentially identical in the \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) and wild-type cells (Figure 2B), indicating that the defect was specific for cytokinesis and that the other stages of the cell cycle were apparently normal. At steady state, less than 15% of the \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cells were mononucleated, whereas over 75% had six or more nuclei (Figure 2C). The \( \text{p}\text{i}3k^{1^{-2}} \) and \( \text{pten}^{-} \) cultures also contained multinucleated cells. About 20% had up to five nuclei, and about 3% contained six to ten. In contrast, nearly 75% of the wild-type cells were mononucleated, and none had more than five nuclei. We also determined the distribution of nuclei in cultures of \( g\text{j}^{3} \) cells, which lack the unique \( g\text{j}^{3} \) subunit of heterotrimeric G proteins found in \( D. \text{discoideum} \). Interestingly, these populations contained over 95% mononucleated cells, suggesting that G protein signaling may interfere with cytokinesis (data not shown).

We also examined the cells grown on plastic surfaces, which as described above, is a less stringent condition than suspension growth (Uyeda et al., 2000). Cells lacking important components for cytokinesis such as myosin II, cortexillin, or Rac E, for example, which cannot grow in suspension, are often able to divide on surfaces. Likewise, the \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cells were able to grow under this condition. On surfaces, the \( \text{p}\text{i}3k^{1^{-2}}, \text{pten}^{-}, \) and \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cells still contained abnormally sized cells (Figures 2D–2F and data not shown; Movies S6 and S11 shows a \( \text{pten}^{-} \) cell and a \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cell failing cytokinesis). Fields of the \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cultures examined by phase microscopy contained cells with diameters up to 120 \( \mu \text{m} \). Expression of PTEN-GFP or human PTEN-YFP (hPTEN-YFP) in the \( \text{p}\text{i}3k^{1^{-2}}\text{pten}^{-} \) cells completely reversed these defects (Figure 2D). PTEN_{16-512}-YFP and PTEN_{C124S}-YFP did not eliminate the large-cell phenotype (data not shown).
Figure 2. Analysis of Growth in Wild-Type and Mutant Cell Lines

(A) Curves show growth of shaking cultures of wild-type (blue diamonds), \textit{pi3k}^{1-2−} (pink squares), \textit{pten}− (purple triangles), and \textit{pi3k}^{1-2−}\textit{pten}− (green circles) cells. All values in an experiment were normalized to the wild-type value at day 4, which was typically 1–2 × 10^7 cells/ml. Experiments were repeated four times, and averages and standard deviations are shown. Nomarski DIC images show typical wild-type (upper) and \textit{pi3k}^{1-2−}\textit{pten}− (lower) cells after 3 days of shaking culture. The scale bar is 15 μm.

(B) Wild-type and \textit{pi3k}^{1-2−}\textit{pten}− cells were grown in shaking conditions. Cells and DAPI-stained nuclei were counted. Wild-type cells (dark blue), wild-type nuclei (light blue), \textit{pi3k}^{1-2−}\textit{pten}− cells (dark green), \textit{pi3k}^{1-2−}\textit{pten}− nuclei (light green).

(C) Wild-type (dark blue), \textit{pi3k}^{1-2−} (pink), \textit{pten}− (purple), and \textit{pi3k}^{1-2−}\textit{pten}− (green) cultures were shaken for 3 days, plated, and stained with DAPI. Random fields were photographed and fractions of cells containing the designated number of nuclei were determined. Averages of three experiments comprising over 200 cells for each line are shown. Selected epifluorescence images of mononucleated wild-type and multinucleated \textit{pi3k}^{1-2−}, \textit{pten}−, and \textit{pi3k}^{1-2−}\textit{pten}− cells are shown. The scale bars are 10 μm.

(D) Cells adhering to a coverslip were grown for 3 days and were imaged by 400× phase contrast microscopy. Typical fields of wild-type, \textit{pi3k}^{1-2−}\textit{pten}−, as well as PTEN-GFP or hPTEN-YFP expressed in \textit{pi3k}^{1-2−}\textit{pten}− cells, are shown. The scale bar is 10 μm.

(E) Random fields of DAPI-stained cultures of the indicated cell lines grown on surfaces. Insets show enlargements of selected portions of each field. The scale bar is 30 μm.

(F) Cultures of wild-type (dark blue), \textit{pi3k}^{1-2−}\textit{pten}− (maroon), PTEN/\textit{pi3k}^{1-2−}\textit{pten}− (yellow), hPTEN/\textit{pi3k}^{1-2−}\textit{pten}− (light blue), hPTEN_{16-515}/\textit{pi3k}^{1-2−}\textit{pten}− (purple), and PTEN_{C124S}/\textit{pi3k}^{1-2−}\textit{pten}− (salmon) cells were grown on surfaces and stained with DAPI. Fractions of cells with designated numbers of nuclei are shown. Averages of two experiments comprising over 200 cells for each line are shown.

We also perturbed PI(3,4,5)P₃ levels in wild-type and \textit{pten}− cells by addition of the PI3K inhibitors LY294002 and wortmannin. We searched for cells entering or in the midst of the cytokinesis process, added the drugs, and observed the immediate effects. The need for imaging restricted our studies to cells on surfaces on which cytokinesis defects are typically less penetrant (see Figure 2). Nevertheless, about 25% of the events showed; see below). We have previously shown that PTEN_{16-515}-YFP, which lacks the N-terminal PI(4,5)P₂ binding motif, fails to interact with the membrane or rescue the chemotaxis defect of the \textit{pten}− cells. \textit{PTEN}C_{124S}-YFP displays low catalytic activity, but it does interact with the membrane and partially rescues the aggregation defect (Iijima et al., 2004). The human counterparts of these mutants, hPTEN_{1-403}-YFP, which does not interact with the membrane (unpublished data), and catalytically inactive hPTEN_{C_{124S}}-YFP were ineffective in reversing the large-cell phenotype. DAPI staining showed that \textit{pi3k}^{1-2−}\textit{pten}− cells grown on surfaces were a mixed population containing mononucleated cells as well as multinucleated cells containing up to 100 nuclei (Figure 2E). The mononucleated cells derived from traction-mediated cytofission as well as cytokinesis (data not shown; see below). Expression of PTEN-GFP or hPTEN-YFP yielded primarily mononucleated cells, whereas hPTEN_{1-403}-YFP did not eliminate the multinucleated cells. \textit{PTEN}C_{124S}-YFP shifted the distribution toward mononucleated cells but did not eliminate the multinucleated phenotype. Quantification of the distributions of nuclei in the wild-type and \textit{pi3k}^{1-2−}\textit{pten}− cells and the effects of expression of PTEN-GFP, hPTEN-YFP, hPTEN_{1-403}-YFP, and \textit{PTEN}C_{124S}-YFP in the \textit{pi3k}^{1-2−}\textit{pten}− cells are shown in Figure 2F.
were perturbed by either drug treatment (Table 1). The cells either failed to divide, or the process was extremely delayed. This failure rate on surfaces is comparable to that observed for cells lacking myosin II (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). Examples of the failed attempts at cytokinesis in wild-type and pten− cells are included as Movies S7 and S9.

Cells Lacking Dual PI3K/PTEN Regulation Are Unable to Respond to External Cues

We reasoned that our genetic perturbations eliminated modulation of PI(3,4,5)P3 levels by internal cues operating during cytokinesis and that this lack of regulation would also be reflected in the temporal response to chemoattractant. We examined the regulation of PI(3,4,5)P3 levels during stimulation with the growth-stage chemoattractant, folic acid. As shown in Figure 3, wild-type cells responded to a uniform increase in folic acid with a transient production of PI(3,4,5)P3. While no PI(3,4,5)P3 was observed in the pi3k1-2− cells in this assay, more sensitive assays have shown that PI(3,4,5)P3 levels are low but can still be modulated in differentiated pi3k1-2− cells stimulated with cAMP (Huang et al., 2003; Meili et al., 2000). As previously shown in the pten− cells, PI(3,4,5)P3 levels were elevated in the absence of stimulation, and the response to folic acid was larger and more prolonged than in the wild-type cells, as has been observed previously for CAMP stimuli (Iijima and Devreotes, 2002). As anticipated, in the pi3k1-2−-pten− cells, basal levels were also elevated, but exposure to folic acid had no further effect. Differentiated pi3k1-2−-pten− cells also had high uniform levels of membrane PI(3,4,5)P3 that were insensitive to cAMP stimulation (data not shown). To assess the integrity of the signaling system, we also stimulated pi3k1-2−-pten− cells expressing the isolated membrane targeting domain of PI3K2 (N600-GFP) and found that it translocated normally to the membrane.

We quantified these responses in resting cells and at 5 and 30 s after stimulus addition by obtaining the ratio of signal within a small region on the membrane and a region of the cytosol. The changes in the ratios as a function of time reflected the changes seen in the images. In the wild-type and pi3k1-2− cells, the “basal” ratios were low, as is apparent from the images. In the pten− and pi3k1-2−-pten− cells, the prestimulus ratios were significantly elevated and were similar to each other. The higher basal PI(3,4,5)P3 levels correlated with a higher level of membrane protrusions including an unusual number of PI(3,4,5)P3-labeled filopodia. Images of the wild-type and pi3k1-2−-pten− cells expressing

| Table 1. Defective Cytokinesis Induced by PI3K Inhibitors |
|------------|------------|------------|----------------|
|            | Total | Fail | Delayed | Defective |
| Wild-Type  |       |      |         |           |
| Control    | 30    | 0    | 0       | 0%        |
| DMSO       | 16    | 0    | 1       | 6%        |
| LY294003   | 28    | 5    | 4       | 32%       |
| pten−      |       |      |         |           |
| Control    | 37    | 1    | 0       | 3%        |
| DMSO       | 21    | 0    | 2       | 9%        |
| LY294003   | 31    | 3    | 4       | 23%       |
| Wortmannin | 20    | 2    | 3       | 25%       |

Figure 3. PI(3,4,5)P3 Accumulation in Wild-Type and Mutant Cells during Folic Acid Stimulation
(A–D) (A) Wild-type, (B) pi3k1-2−, (C) pten−, and (D) pi3k1-2−-pten− cells expressing PHcrac-GFP were stimulated with a uniform concentration (10 μM) of folic acid. The scale bar is 5 μm. Images and plots of the membrane:cytosol ratio of each cell line 20 s before the addition of folic acid, during the peak response at 10 s, and after 40 s of stimulation are shown. A box centered on the membrane also encompasses a portion of the cytosol. We therefore estimated the membrane signal by subtracting 50% of the cytosolic value from the value in the box centered on the membrane. Each time point indicates the average and standard error of six cells from two independent experiments.
PHCrac-GFP and examined by total internal reflection fluorescence microscopy (TIRFM) are included as Figure S2. The effects of these mutations on PI(3,4,5)P₃ levels are consistent with direct measurements of PI3K and PTEN activities (see Discussion).

Asymmetric PI(3,4,5)P₃ Distribution Influences Localization of Cytoskeletal Proteins

To further investigate the defects in pi3k¹⁻²⁻pten⁻⁻ cells, we imaged the distribution of Coronin-GFP, an actin binding protein that labels actin-based projections at the front of migrating cells and at the poles of dividing cells (Gerisch et al., 1995); GFP-myosin II, which accumulates in the furrow of dividing cells and plays a major role in cytokinesis (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987); as well as PHCrac-GFP. Figure 4 illustrates striking examples in which furrow formation fails between some dividing nuclei and succeeds between others. Coronin-GFP was normally associated with ruffles along the cell cortex in migrating pi3k¹⁻²⁻pten⁻⁻ cells. During cytokinesis, Coronin-GFP is suppressed in the midzone and furrows. However, in furrows that fail, Coronin-labeled ruffling reappears as the constriction is released (Figure 4A and Movie S9). In contrast, GFP-myosin II concentrated in forming furrows, but then it suddenly dispersed as the furrow was replaced with cortical ruffles on the forming binucleated cell (Figure 4B). Thus, the failure of Coronin-GFP suppression and GFP-myosin II concentration in the furrow both correlated strongly with aborted cytokinesis. For cells on surfaces, about 40% of the failed furrows aborted in this manner (n = 37). This experiment is included as Movie S10. We never observed a furrow that failed to properly localize the cytoskeletal proteins and succeeded to divide. On the other hand, in 60% of failed furrows, cytoskeletal proteins localized properly, but then the cell failed to sever the narrow bridge connecting the two daughters. Eventually, the bridge increased its diameter and the two cells flowed back together (Movie S10). This phenotype resembles the kinesin Kif12 mutants recently described (Lakshmikanth et al., 2004). In contrast to the cytoskeletal proteins, PI(3,4,5)P₃ remained uniformly distributed along the cell perimeter (Figure 4C), whether the pi3k¹⁻²⁻pten⁻⁻ cells succeeded or failed to divide. Movie S11 shows an example of a cytokinesis failure in pi3k¹⁻²⁻pten⁻⁻ cells expressing PHCrac-GFP.

Localization of PI3K and PTEN Depends on Mitosis

To test the role of the spindle microtubules in the regulation of PI(3,4,5)P₃ during cytokinesis, we treated cells expressing PHCrac-GFP or PTEN-GFP with the mitotic inhibitor nocodazole. As previously shown, the inhibitor had little effect on motility but delayed cells at mitosis (Kitanishi-Yumura and Fukui, 1987; Graf et al., 2003). PI(3,4,5)P₃ accumulation on ruffles during random migration was normal, suggesting that the regulation of PI3K and PTEN was unperturbed. As cells rounded-up to enter cytokinesis and the ruffles subsided, PI(3,4,5)P₃ disappeared, PHCrac-GFP accumulated in the cytosol, and PTEN-GFP became uniformly associated with the membrane. These early events were indistinguishable in treated or untreated cells (compare Figure 1 and Figure 5). However, whereas in untreated cells, this quiescent stage lasted less than 1 min, in nocodazole, it persisted. After an hour, about 10% of the treated cells, compared with only 0.5% of untreated cells, displayed this profile. When the nocodazole was washed out, the arrested cells resumed cytokinesis and PI(3,4,5)P₃, PI3K-GFP, and PTEN-GFP localized appropriately (Figure 5B and data not shown).

Discussion

Our results show that an elegant regulatory circuit involving phosphoinositide signaling, similar to the sys-
affect migration. In cytokinesis, we find strikingly parallel redistributions of PI3K and PTEN. As cells round at the onset, PTEN moves uniformly to the membrane. Then, as the cell elongates, PI3K and PTEN associate with the membrane at the poles and furrow, respectively (Figure 6). Our disruption of both PI3K and PTEN activities generated cells in which PI(3,4,5)P3 could not be modulated. This led to a specific failure in cytokinesis, resulting in large, multinucleated cells. Expression of active PTEN from D. discoideum or human reversed the defect. This is the first report that regulation of PI(3,4,5)P3 metabolism plays a role in cytokinesis. However, considering the general role of PI(3,4,5)P3 in migration, it is likely that the mechanism we describe here for cytokinesis will be found in other organisms (Hannigan et al., 2004; Wang et al., 2002). A recently reported association of PTEN with the septa of S. pombe may be related (Mitra et al., 2004).

Based on studies of migrating cells, we can speculate on the role of PI(3,4,5)P3 in cytokinesis. In a migrating cell, there is a steep internal gradient of PI(3,4,5)P3 that promotes actin-filled projections selectively at the labeled membrane protrusions (arrows), while other cells (brackets) stall at the G2/M transition and are quiescent. The right panel shows the localization of PTEN-GFP at the rear of interphase cells (arrows) and the uniform accumulation of PTEN-GFP at the membrane of a cell stalled at entry to mitosis (bracket). The scale bar is 5 \( \mu\text{m} \).

(B) PTEN-GFP cells were treated for 3 hr with nocodazole, washed, and observed for the times indicated. Cells underwent cytokinesis, and PTEN-GFP localized normally.

Figure 6. Roles of the “Phosphoinositide-Linked Polarity Circuit” in Cytokinesis and Chemotaxis
(A) Left, top: current models envision a reciprocal interaction between the spindle microtubules and cytoskeletal dynamics that work together to form the furrow and drive cytokinesis. Evidence for and against the roles of the depicted interactions in various cell types have been extensively reviewed (Guertin et al., 2002; Oegema and Mitchison, 1997). Left, bottom: The scheme proposed here introduces the additional participation of membrane phosphoinositides. Phosphoinositide metabolism establishes the rear of a migrating cell. This absence of PI(3,4,5)P3 from the furrow inhibits actin-based projections in this region and may permit the acto-myosin-based contractions to occur. This segregation of cytoskeletal activities is consistent with previ-
ous studies of normal rat kidney cells in which local application of actin polymerization inhibitors to the poles prevents cytokinesis, whereas treatment of the furrow accelerates the process (O’Connell et al., 2001). It is also possible that concurrent elevation of PI(4,5)P_2 in the furrow may contribute to the polarization of the cell (Brill et al., 2000). In fact, PTEN contains a PI(4,5)P_2 binding motif that could possibly direct it to the furrow of a dividing cell or the back of a migrating cell. However, we have no direct evidence that PTEN actually binds to PI(4,5)P_2 or that this phosphoinositide is elevated in these regions.

The inability to regulate PI(3,4,5)P_3 and influence the activity of the cytoskeleton in the pi3k^1−2−pten cells likely contributes to the observed failure at multiple stages of cytokinesis. First, it would be more difficult for these cells to reset polarity at the onset of cytokinesis. Second, the uniform distribution of PI(3,4,5)P_3 would be unable to reinforce the segregation of cytoskeletal activities during furrow formation. In fact, in the pi3k^1−2−pten cells imaged on surfaces, nascent furrows lacking Coronin-GFP and with accumulated GFP-myosin II initially formed, but then, the myosin was often lost, and the furrow was replaced with Coronin-labeled, actin-based ruffles. While we could not image under shaking conditions in which the pi3k^1−2−pten cells always fail cytokinesis, we presume that the role of PI(3,4,5)P_3 to properly localize and stabilize the cytoskeletal proteins is critical. The fact that even the pi3k^1−2−pten cells were able to occasionally divide when grown on surfaces is not surprising since cells lacking other critical components that mediate cytokinesis, including myosin II, cortexillin, and Rac E, display the same phenotype. While the rate of the cell cycle was unaffected in the pi3k^1−2−pten cells, it is likely that they are defective in additional processes, such as macropinocytosis and phagocytosis, which require PI(3,4,5)P_3 regulation of the cytoskeleton.

Our disruption of PTEN in a cell already lacking PI3K1 and PI3K2 removed most of the temporal and spatial regulation of PI(3,4,5)P_3, but it did not eliminate the basal levels. It has been shown directly that PI3K1 and PI3K2 contribute most of the regulated, but little of the basal, PI3K activity (Huang et al., 2003). An additional class I and a class III PI3K (PIK3 and PIK5, respectively) have been previously reported (Meili et al., 1999; Zhou et al., 1995). Our observation prompted us to reexamine the database for additional PI3Ks. We found that the database includes four additional enzymes. An analysis of the PIKs, together with suggested nomenclature for the new enzymes, is included as Figure S4. Presumably, some of these PI3Ks contribute the basal activity. A constant source of PI(3,4,5)P_3 synthesis could explain the observations in Figure 3 as follows: in wild-type and pi3k^1−2− cells, the basal levels of PI(3,4,5)P_3 are held in check by PTEN, and the transient dissociation of PTEN from the membrane contributes to the modulation of PI(3,4,5)P_3. When PTEN is deleted, basal levels increase, but modulation of PI(3,4,5)P_3 is still supplied by activation of PI3K1 and PI3K2. But in pi3k^1−2− cells, only the unchecked constant basal levels remain.

Our experiments with nocodazole suggest that events depending on the spindle microtubules control the spatially localized activities of PI3K and PTEN. As cells round-up at the onset of cytokinesis, PTEN and myosin II associate uniformly with the membrane, and PI(3,4,5)P_3 signaling is suppressed. It is also known that the microtubule network is disassembled at this stage (Neujahr et al., 1998). Cells treated with nocodazole are delayed at this stage because the spindle cannot be easily constructed. Under these conditions, the polarization of PI3K and PTEN, PI(3,4,5)P_3 signaling, and membrane ruffling are concomitantly delayed. This suggests the existence of a checkpoint that prevents further progression into cytokinesis if the spindle fails to assemble. We speculate that the elongation of the spindle leads to the localization of PI3K and PTEN to the membrane at the poles and furrow, respectively. Perhaps specific proteins link ends of the astral microtubules to the actin network (Canman and Bement, 1997; Goode et al., 2000; Hestermann et al., 2002). For directed migration, the upstream control derives from G protein-coupled receptor (GPCR) activation, whereas cytokinesis clearly does not require G protein signaling and is driven by intrinsic cues (Devreotes and Zigmond, 1988; Guertin et al., 2002). Yet, in both cases, the signals somehow trigger local membrane binding of PI3Ks and PTEN. Since the two processes share these elements, it is possible that the pathways may interact at the level of membrane binding sites for PI3K and PTEN or at targets for PI(3,4,5)P_3. We have noted that shaking cultures of wild-type cells contain about 25% multinucleated cells and that these multinucleated cells are absent from gß− cultures. This may point to an antagonism between the GPCR signaling and cytokinesis pathways. Other recent reports have suggested undefined positive or negative roles for G protein signaling in cytokinesis (Fuse et al., 2003; Grill et al., 2003; Manning, 2003).

It is well known that defects in regulation of PI(3,4,5)P_3 are a major determinant in cancer (Phillips et al., 1998; Vivanco and Sawyers, 2002). One common mechanism of elevating PI(3,4,5)P_3 levels is by inactivation of the tumor suppressor PTEN. Mutations in PTEN are found in a large fraction of human cancers (Vazquez and Sellers, 2000). Recent evidence also has shown that mutations in PIK3CA, which increase kinase activity, are found in many colorectal tumors (Samuels et al., 2004). The proposed role of elevated PI(3,4,5)P_3 is to promote cell growth and survival. Other experiments show that activation of cell motility occurs with elevated PI(3,4,5)P_3 levels and may play a role in tumor progression (Chung et al., 2004; Wu et al., 2003). Our findings presented here suggest yet another potential role for the phosphoinositide in cancer. Although elevation of PI(3,4,5)P_3 levels does make cells grow faster, perturbation of its proper regulation may also promote disease by interfering with proper cell division. Moreover, defects in cytokinesis may also lead to defects in chromosome integrity, leading to aneuploidy, commonly found in many cancers. Many cancers appear to progress most rapidly once cells become aneuploid (Bose et al., 2002). In this regard, we have noted that a small fraction of the pi3k^1−2− cells have extremely enlarged nuclei and unbalanced nuclear divisions. Thus, disruption of a PI(3,4,5)P_3 polarity circuit regulat-
ing cytokinesis like that described here may significantly contribute to tumor progression.

Experimental Procedures

Cell Growth and Development

All cell lines were cultured axenically in HL5 medium (Huang et al., 2003) at 22°C. Transformants were maintained in 20 μg/ml G418, 30 μg/ml Hygromycin, or both as required. Cells stimulated with folic acid were grown on nutrient SM agar plates with Klebsiella aerogenes (Janetopoulos et al., 2004).

Construction of \( \text{p3k}^{1-2-} \text{pten} \) Cells

To construct the PTEN disruption vector, the Hygromycin resistance cassette was inserted into the BamHI site in the PTEN coding region and cloned into the TA cloning vector pCR2.1 (Invitrogen). The linearized disruption vector (10 μg) was introduced into growth-phase \( \text{p3k}^{1-2-} \) cells (Funamoto et al., 2001).

Vector Constructs

HPTEN-YFP and deletion mutants fused to YFP were amplified by PCR from pSG5-L-HA-PTEN;WT (Vazquez et al., 2000) and cloned into pJX1.

Microscopy

Wide-field epifluorescence microscopy images of DAPI-stained and living cells were obtained by using an inverted Zeiss Axiovert Drechsel, D.N., Hyman, A.A., Hall, A., and Glotzer, M. (1997). A Microscopy Vector Constructs 100 TV with a 40× 1.3 N.A. Achrostigmat lens and were collected requirement for Rho and Cdc42 during cytokinesis in pi3k content/full/8/4/467/DC1.

Supplemental Data including figures and movies referred to in the text are available at http://www.developmentalcell.com/cgi/trimeric G proteins regulate daughter cell size asymmetry in the leading edge of the cytokinetic bridge formation during germ line cytokinesis. Development 127, 3855–3864.

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