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PtdIns(4,5)P2 Functions at the Cleavage Furrow during Cytokinesis

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Summary

Phosphoinositides play important roles in regulating the cytoskeleton and vesicle trafficking, potentially important processes at the cleavage furrow. However, it remains unclear which, if any, of the phosphoinositides play a role during cytokinesis. A systematic analysis to determine if any of the phosphoinositides might be present or of functional importance at the cleavage furrow has not been published. Several studies hint at a possible role for one or more phosphoinositides at the cleavage furrow. The best of these are genetic data identifying mutations in phosphoinositide-modifying enzymes (a PtdIns(4)P-5-kinase in *S. pombe* [1, 2] and a PI-4-kinase in *D. melanogaster* [3]) that interfere with cytokinesis. The genetic nature of these experiments leaves questions as to how direct may be their contribution to cytokinesis. Here we show that a single phosphoinositide, PtdIns(4,5)P2, specifically accumulates at the furrow. Interference with PtdIns(4,5)P2 interferes with adhesion of the plasma membrane to the contractile ring at the furrow. Finally, four distinct interventions to specifically interfere with PtdIns(4,5)P2 each impair cytokinesis. We conclude that PtdIns(4,5)P2 is present at the cleavage furrow and is required for normal cytokinesis at least in part because of a role in adhesion between the contractile ring and the plasma membrane.

Results and Discussion

PtdIns(4,5)P2 Localizes to the Cleavage Furrow

We imaged the subcellular localization of the phosphoinositides by using spectral variants of Enhanced Green Fluorescent Protein (EGFP) fused to specific binding domains. In particular, we could detect PtdIns(3)P by using p40PX-EYFP [4], PtdIns(4)P by using FAPP1-PH-EYFP [5], PtdIns(3,4)P2 by using TAPP1-PH-EYFP [5], PtdIns(4,5)P2 by using PLCδ-PH-EGFP [6], and PtdIns(3,4,5)P3by using BTK-PH-EGFP [7]. There do not yet exist validated reporters for PtdIns(5)P or PtdIns(3,5)P2. We expressed each reporter in NIH3T3 fibroblasts at comparable levels (data not shown) and performed time-lapse fluorescence microscopy of complete cell cycles unperturbed by drugs or other manipulations. Live imaging to track the division of each cell into two daughters allowed unambiguous identification of the cleavage furrow. We observed that the PtdIns(4,5)P2 reporter accumulated at the furrow (Figure 1A and Movie S1 in the Supplemental Data available with this article online), whereas the other reporters did not (Movies S2–S5). This observation was replicated in RAW mouse macrophages, HeLa human cervical endothelial cells, and CHO (Chinese hamster ovary) cells (Figure 1A and Movie S6). Accumulation of PtdIns(4,5)P2 was observed from initial furrow invagination to abscission.

Some PH domains may bind proteins instead of, or in addition to, lipids. To verify that we were truly observing PtdIns(4,5)P2, we used an independent reporter. The Tubby protein's C-terminal Tubby domain has a sequence and structure unrelated to PH domains but specifically binds PtdIns(4,5)P2 in vitro and in vivo [8]. We observed GFP-TubbyC subcellular localization during cytokinesis in CHO (Figure 2B) and HeLa cells (Movie S7). This reporter also localized to the furrow. A nonbinding triple-point mutant (K330A, K339A, K383A) [8], GFP-TubbyC-KKK, failed to localize (data not shown). Given that two distinct and unrelated reporters for PtdIns(4,5)P2 each accumulate at the furrow, whereas a nonbinding mutant does not, we conclude that PtdIns(4,5)P2 accumulates at the cleavage furrow.

Accumulation of PtdIns(4,5)P2 at the furrow could represent specific accumulation of PtdIns(4,5)P2 or of total membrane [9]. We used a membrane marker, EGFP fused to the c-Ha-Ras farnesylation signal (EGFP-F), and compared its localization to that of PLCδ-PH-EGFP and GFP-TubbyC, or directly compared it to that of PLCδ-PH-TDRFP in the same cell. All assessments yielded similar results, showing a 2-fold (2.2 ± 0.1, n = 8) increase in total membrane and a 9-fold (8.9 ± 2.2, n = 8) increase in PtdIns(4,5)P2; this represents a net 4.5-fold increase in PtdIns(4,5)P2 concentration at the furrow (Figure 1C). We also observed live CHO cells by spinning-disk confocal microscopy (Figures 1A and 1B). Within a confocal plane, we detected a 5.6 ± 1.6-fold (n = 4) increase in PtdIns(4,5)P2 concentration at the furrow (data not shown). We conclude that there is a specific increase in the concentration of PtdIns(4,5)P2 at the cleavage furrow. We also note that none of the other phosphoinositides accumulate at the furrow (Supplementary Information, Movies S2–S5).

PtdIns(4,5)P2 Functions in Adhesion of the Plasma Membrane to the Contractile Ring

We hypothesized potential functions, including assembly of the contractile ring, adhesion of the ring to the plasma membrane, or vesicle trafficking, for PtdIns(4,5)P2 at the furrow. To explore these possibilities, we perturbed PtdIns(4,5)P2 to determine the effect

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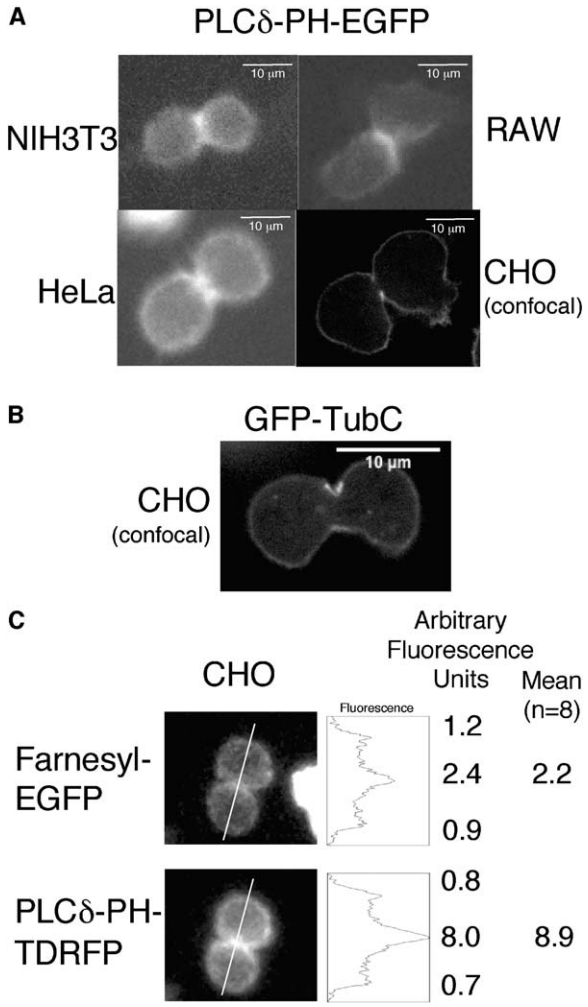

Figure 1. PtdIns(4,5)P2 Accumulates at the Cleavage Furrow during Cytokinesis

(A) The PtdIns(4,5)P2 reporter PLCδ-PH-EGFP was expressed and imaged by time-lapse wide-field or spinning-disk confocal microscopy in NIH3T3 fibroblasts, RAW macrophages, HeLa cells, or CHO cells. A representative cell undergoing cytokinesis is shown for each. See also Movie S1.

(B) Another reporter for PtdIns(4,5)P2, GFP-TubbyC, was expressed and imaged in CHO cells via spinning-disk confocal microscopy. A representative cell undergoing cytokinesis is shown. See also Movie S7.

(C) PtdIns(4,5)P2 concentrates at the furrow, more so than total membrane. Shown is a dividing CHO cell expressing farnesylated EGFP (marking the membrane), and PLCδ-PH-TDRFP (marking PtdIns(4,5)P2) with intensity profile plots. On average, total membrane increases 2.2- ± 0.1-fold (n = 8; numbers represent mean ± standard error) at the furrow, significantly less than the 8.9- ± 2.2-fold (n = 8) increase in PtdIns(4,5)P2.

on cytokinesis, first by overexpressing the PLCδ-PH or Tubby domains to specifically sequester PtdIns(4,5)P2. We transfected low or 10-fold-higher levels of EGFP-F, PLCδ-PH-EGFP, or GFP-TubbyC, and after 24-48 hr processed the cells to visualize F-actin (Texas Red-phalloidin) and DNA (DAPI). Low or high levels of EGFP-F or low levels of PLCδ-PH-EGFP or GFP-TubbyC (these do not interfere with cytokinesis; see below) do not interfere with the expected colocalization of F-actin and the plasma membrane (165 out of 165 cells scored, Figure 2). With 10-fold-higher expression (determined by relative fluorescence intensity), levels that do interfere with cytokinesis (see below), 7.2% of cells (11 of 152) develop separation of F-actin from the plasma membrane during cytokinesis. We also used the PtdIns(4,5)P2-phosphatase synaptojanin. In control experiments, synaptojanin reduced PtdIns(4,5)P2 levels by 45% (data not shown). When synaptojanin was coexpressed with low levels of EGFP-F, we again observed detachment of F-actin from the plasma membrane at the furrow (11.5%, 3 of 26 cells).

This suggested that PtdIns(4,5)P2 functions to mediate the interaction between the contractile ring and the plasma membrane. Presumably there exist one or more mechanisms of adhesion to ensure that, when the ring contracts, the membrane comes along. Although prior studies have not identified plasma-membrane components that mediate this adhesion, our data suggested that PtdIns(4,5)P2 might. We wondered whether we could detect evidence of decreased adhesion upon blockade of PtdIns(4,5)P2. Raucher et al. used optical tweezers to measure the force required to tug on the plasma membrane in interphase cells [10] and found a precipitous drop upon blockade of PtdIns(4,5)P2 by the PLCδ-PH domain. However, these measurements are not feasible at the furrow because of spatial and temporal constraints. Instead, we reasoned that movement of the plasma membrane at the furrow will largely reflect the balance between outward osmotic pressure and inward tension from adhesion to the cytoskeleton [10]. Therefore, switching from isotonic to hypotonic conditions should cause expansion of the plasma membrane at a rate reflecting the strength of adhesion to the cytoskeleton.

We synchronized cells by washout from monastrol arrest. During cytokinesis, the media were switched, and furrow diameter was measured over time. As expected, switching to hypotonic conditions caused expansion of the plasma membrane at the furrow (see Figure 3A). We quantified the rate of expansion and compared the effects of various perturbations. As predicted, destabilization of F-actin by latrunculin B increased hypotonic expansion of the plasma membrane at the furrow (see Figures 3B and 3D). Conversely, the actin-stabilizing agent jasplakinolide prevented expansion of the furrow. Agreement with the results of Raucher et al. [10] serves to validate our assay.

We next measured the effect of PtdIns(4,5)P2 blockade on adhesion. High levels of PLCδ-PH-EGFP or GFP-TubbyC each significantly increased the rate of hypotonic expansion (see Figures 3C and 3D). Controls, including GFP-TubbyC-KKK, EGFP-F, BTK-PH-EGFP, and TAPP1-PH-EYFP, each had no effect. Taken together these data suggest that PtdIns(4,5)P2 functions to promote adhesion of the plasma membrane to the actin cytoskeleton at the cleavage furrow.

PtdIns(4,5)P2 Is Required for Normal Cytokinesis

We next addressed whether PtdIns(4,5)P2 is required for normal cytokinesis. We noted that high levels of PLCδ-PH-EGFP caused cells to become transiently

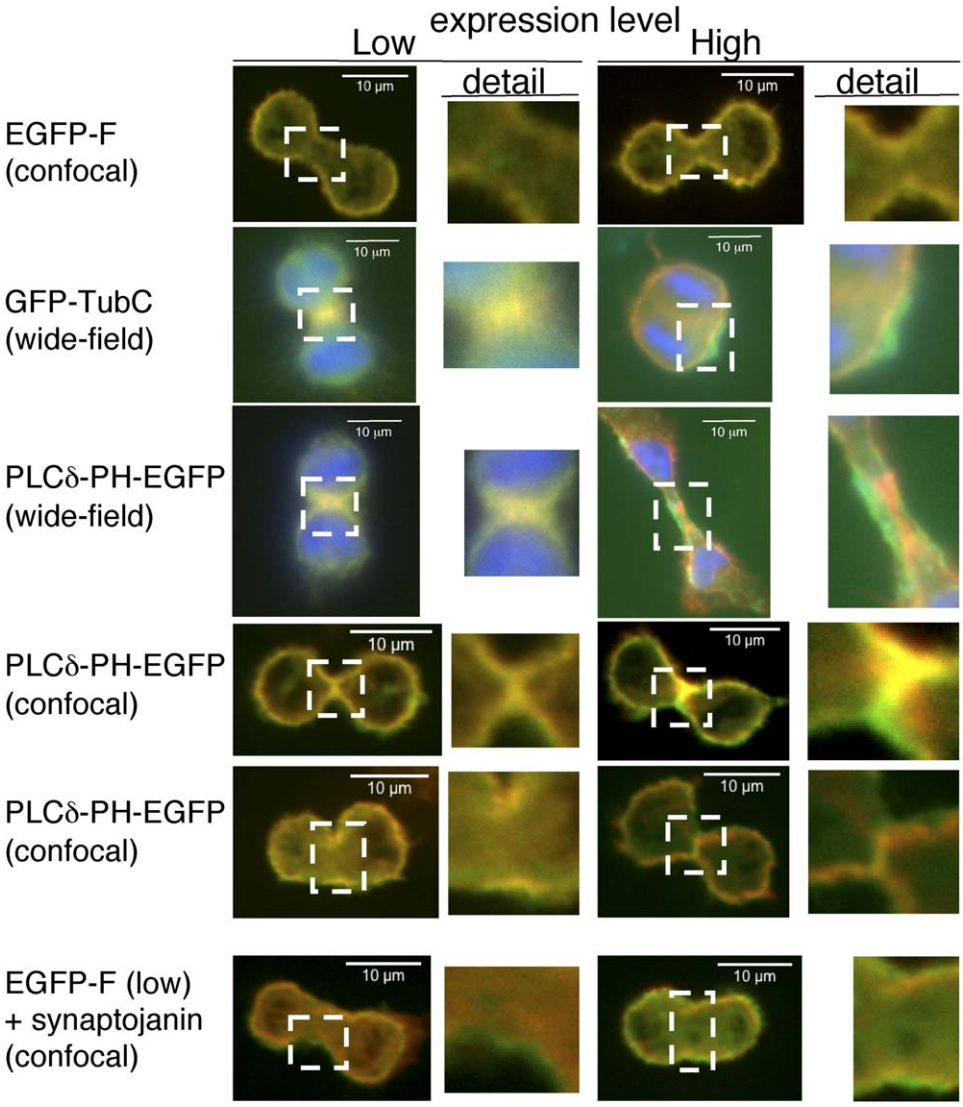

Figure 2. Interference with PtdIns(4,5)P2 Causes Separation of the Plasma Membrane from the Contractile Ring

CHO and HeLa cells were transfected with low or 10-fold-higher levels of farnesylated EGFP (EGFP-F), PLCδ-PH-EGFP or GFP-TubbyC expression vectors. Twenty-four hours later, cells were fixed and stained with Texas Red-phalloidin and DAPI. Images were taken by wide-field (HeLa cells) or spinning-disk confocal microscopy (CHO cells). Cells in cytokinesis were identified by chromatin pattern and the presence of a cleavage furrow. A magnified view of the dashed box is provided for each. Low levels of EGFP-F, PLCδ-PH-EGFP, or GFP-TubbyC or high levels of EGFP-F always show the expected co-localization of F-actin and the plasma membrane (165 out of 165 cells). However, high levels of either PtdIns(4,5)P2 binding protein cause separation of F-actin from the membrane in 7.2% of cells (11 of 152). Furthermore, synaptojanin, a PtdIns(4,5)P2-phosphatase, coexpressed with low levels of EGFP-F, also caused separation of F-actin from the plasma membrane at the furrow (11.5%, 3 of 26 cells).

trapped in cytokinesis, quantifiable from the duration of cytokinesis. Compared to BTK-PH-EGFP, FAPP1-PH-EYFP, or EGFP, high levels of PLCδ-PH-EGFP doubled the duration of cytokinesis, as shown in Figure 4A. A similar increase in cytokinesis duration is caused by GFP-TubbyC, unlike GFP-TubbyC-KKK (Figure 4B).

To measure the failure rate of cytokinesis, we measured the fraction of multinucleate cells after 36 hr of expressing equivalently high levels of PLCδ-PH-EGFP or EGFP. PLCδ-PH-EGFP increased the number of multinucleate NIH3T3 (Figure 4C), RAW (data not shown), or 293T cells (Figure 4D) by 3- to 5-fold, demonstrating dramatically increased cytokinesis failure. We manipulated PtdIns(4,5)P2 levels by two additional methods. Expression of a GFP-tagged synaptojanin significantly increased the number of multinucleate 293T cells (Figure 4D). Likewise, expression of a dominant-negative, kinase-inactive PI(4)P-5-kinase-α, the D227A mutant [11], also reduced plasma-membrane PtdIns(4,5)P2 by 46% (data not shown) and significantly increased the number of multinucleate cells (Figure 4D). Therefore, interference by four distinct methods in each case demonstrates a requirement for PtdIns(4,5)P2 in normal cytokinesis.

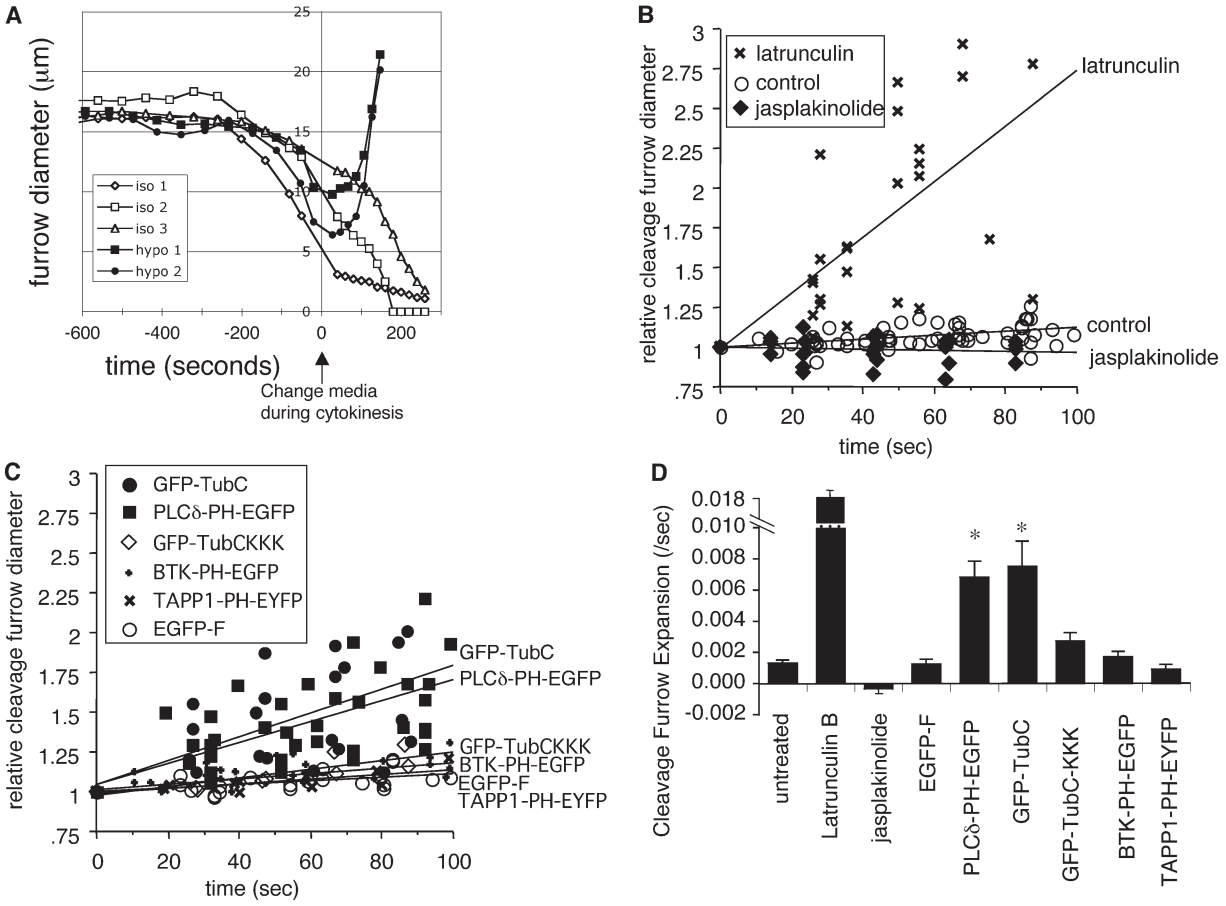

Figure 3. Blockade of PtdIns(4,5)P2 Reduces Plasma Membrane to Cytoskeleton Adhesion

The rate of furrow expansion in response to a rapid drop of tonicity reflects adhesion between the plasma membrane and the contractile ring. (A) Graph of furrow diameter versus time measured for five CHO cells. Cells began cytokinesis in isotonic media, but at time zero they were moved to either isotonic media or water. In isotonic media furrow diameter diminished until abscission (open symbols). In response to hypotonicity, the furrow quickly expanded (closed symbols).

(B) Latrunculin B increases and jasplakinolide reduces the response to hypotonicity at the furrow. Shown are results for CHO cells alone or after 5 μM latrunculin B (to depolymerize actin) or 5 μM jasplakinolide (to stabilize F-actin) was applied before the switch to low tonicity. For each, the best-fit line is shown for normalized furrow diameter versus time. A higher slope indicates faster plasma membrane expansion.

(C) Interference with PtdIns(4,5)P2 reduces adhesion of the plasma membrane to the contractile ring. A comparison of CHO fibroblasts expressing high levels of PLCδ-PH-EGFP, GFP-TubC, GFP-TubCKKK, EGFP-F, BTK-PH-EGFP, or TAPP1-PH-EYFP is shown.

(D) Bar graph indicating the slope and standard error of best-fit lines for (B) and (C). Control relative furrow expansion occurred at 0.0013 ± 0.002 /s (n = 19). Latrunculin B increases it (0.018 ± 0.003/s, n = 8); jasplakinolide reduces it (–0.000 ± 0.0004 /s, n = 7). Each PtdIns(4,5)P2 binding domain (asterisks) significantly increases the rate of expansion (PLCδ-PH 0.007 ± 0.001/s, n = 8, TubC 0.008 ± 0.002/s, n = 6). By contrast, EGFP-F (0.0013 ± 0.0003/s, n = 7), GFP-TubCKKK (0.0027 ± 0.0005/s, n = 3), BTK-PH-EGFP (0.0017 ± 0.0003/s, n = 7), or TAPP1-PH-EYFP (0.0009 ± 0.0003/s, n = 3) have no effect.

The question arises as to whether PtdIns(4,5)P2 is the active molecule or whether it serves as a substrate for PI-3-kinase or phospholipase C. We do not believe that PI-3-kinase functions at the furrow. First, our time-lapse imaging does not detect PtdIns(3,4,5)P3, PtdIns(3,4)P2, or PtdIns(3)P at the furrow (Movies S2, S4, and S5). Second, as shown (Figure 4A), sequestration of PtdIns(3,4,5)P3 does not block cytokinesis. Finally, we observed that wortmannin does not interfere with cytokinesis in CHO cells (data not shown).

We do not have good evidence either for or against a role for phospholipase C. We note that if phospholipase C were to function efficiently at the furrow we would expect depletion of PtdIns(4,5)P2, not the observed enrichment. Furthermore, a recent comprehensive RNAi screen to identify genes required for cytokinesis in *Drosophila* supports a role for PI-4-kinases and PI(4)P-5-kinases, but did not identify any phospholipase C genes required for cytokinesis([12]; U.S. Eggert and C.M. Field, personal communication). The presence at the furrow both of high concentrations of PtdIns(4,5)P2 and of actin binding proteins that bind PtdIns(4,5)P2 [13–17] leads us to favor a direct role for PtdIns(4,5)P2. Nevertheless, we cannot rule out that PtdIns(4,5)P2 might serve an additional role as a substrate for phospholipase C.

The mechanism of linkage between the plasma membrane and the contractile ring has remained an impor-

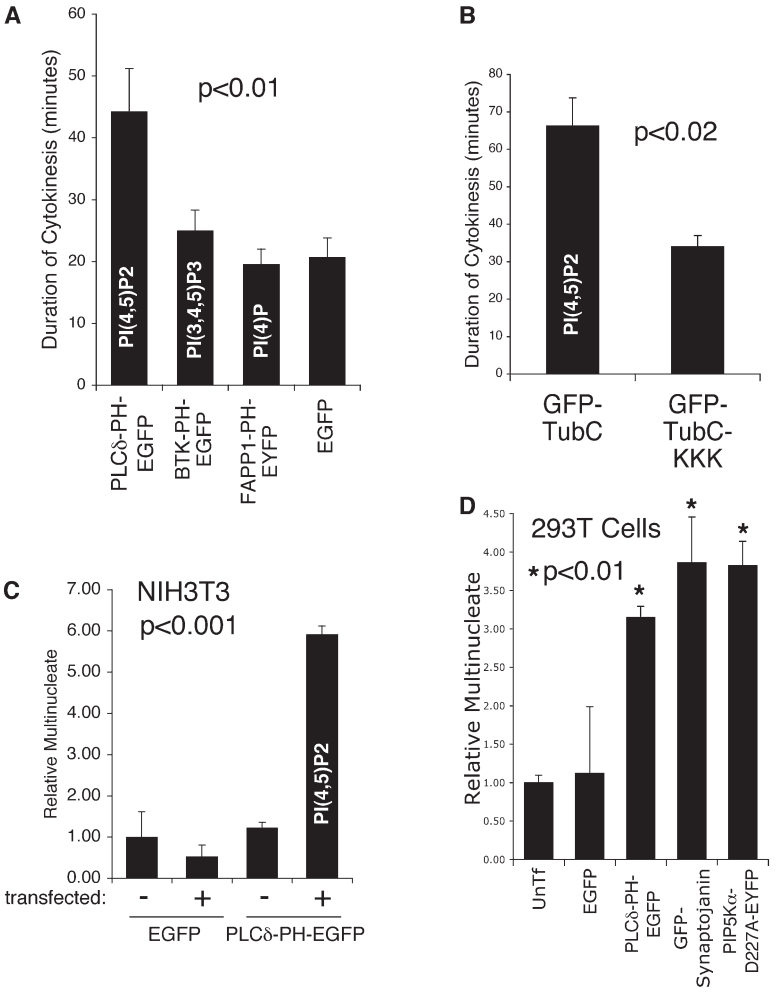

Figure 4. Sequestration of PtdIns(4,5)P2 Impairs Cytokinesis

(A) Cytokinesis duration (initial invagination to abscission) in NIH3T3 cells expressing high levels of PLCδ-PH-EGFP, BTK-PH-EGFP, FAPP1-PH-EYFP, or EGFP. High levels of PLCδ-PH-EGFP double the duration of cytokinesis ($p < 0.01$, unpaired t test, PLCδ-PH-EGFP 44.3 ± 6.9 min, n = 9, BTK-PH-EGFP 25.0 ± 3.3 min, n = 6, FAPP1-PH-EYFP 19.6 ± 2.4 min, n = 5, EGFP 20.7 ± 3.0 min, n = 8, numbers represent mean ± standard error).

(B) Cytokinesis duration in HeLa cells expressing high levels of GFP-TubbyC or GFP-TubbyC-KKK. Cytokinesis duration was as follows: GFP-TubbyC 66.2 ± 7.5 min (n = 11); GFP-TubbyC-KKK 34.0 ± 3.0 min (n = 5), $p < 0.02$ (unpaired t test).

(C) Interference with PtdIns(4,5)P2 causes failure of cytokinesis as measured by accumulation of multinucleate cells. NIH3T3 cells were transiently transfected with high levels of EGFP or PLCδ-PH-EGFP. Thirty-six hours later, the fraction of transfected (EGFP-positive) or untransfected cells that were multinucleate was counted. This fraction is shown relative to the control (untransfected cells from the EGFP plate had 0.9% ± 0.6% multinucleate cells, n = 334). Cells expressing EGFP or untransfected cells from the plate transfected with PLCδ-PH-EGFP had no change in multinucleate cells (0.6- ± 0.2-fold for EGFP, n = 406, 1.2- ± 0.1-fold for untransfected from the PLCδ-PH-EGFP plate, n = 183). However, expression of PLCδ-PH-EGFP caused a 5.9- ± 0.2-fold increase in multinucleate cells (n = 226, $p < 0.001$, unpaired t test).

(D) Measurement of the number of multinucleate cells in cultures of 293T cells either not transfected or transfected with EGFP, PLCδ-PH-EGFP, GFP-synaptojanin, or PI(4)P-5-kinase-α-D227A-EYFP. Of the untransfectec 293T cells, 2.3% ± 0.2% were multinucleate (n = 129). Expression of EGFP had no effect (1.1- ± 0.6-fold increase, n = 187). Interference with PtdIns(4,5)P2 by any of the three latter constructs significantly increased the number of multinucleate cells (PLCδ-PH-EGFP 3.1- ± 0.1-fold increase, n = 180, GFP-synaptojanin 3.8- ± 0.3-fold increase, n = 198, PI(4)P-5-kinase-α-D227A-EYFP 3.8- ± 0.2-fold increase, n = 138, $p < 0.01$, unpaired t test, for cells transfected with each of the latter three versus untransfected).

tant unsolved problem for understanding cytokinesis. Our data suggest a role for PtdIns(4,5)P2 in this linkage. Interference with PtdIns(4,5)P2 might affect this linkage by interfering with actin polymerization. However, we do not see obvious changes in the amount of F-actin at the furrow upon PtdIns(4,5)P2 blockade (data not shown). Instead, in light of the observation that multiple components of the contractile ring can bind PtdIns(4,5)P2, we propose a simple model in which adhesion of the contractile ring to the plasma membrane is mediated by direct interaction of PtdIns(4,5)P2 in the plasma membrane with components of the contractile ring that bind PtdIns(4,5)P2; such components include septin [13], α-actinin [16, 18, 19], profilin [14, 15], and perhaps anillin [20]. Because of the polymeric nature of the contractile ring, each component is found in multiple repeating subunits. Likewise, as we have shown, PtdIns(4,5)P2 is abundant in the plasma membrane at the furrow. Even if each monomeric interaction between a ring protein and PtdIns(4,5)P2 were relatively weak, the tremendous avidity effects in the context of the polymeric ring would be expected to produce an exceptionally strong interaction. An attractive feature of this model is the prediction that release of the contractile-ring components from the plasma membrane at the end of cytokinesis could be accomplished by merely depolymerizing the ring.

This function for PtdIns(4,5)P2 in cytokinesis has an interesting parallel to its function in neurite retraction, which involves plasma-membrane retraction analogous to the ingression that occurs at the furrow. Two groups have described a requirement for PI(4)P-5-kinase and PtdIns(4,5)P2 in neurite retraction [21, 22]. We hypothesize that the similarity between these pathways reflects a conserved mechanism utilizing PtdIns(4,5)P2 for adhesion of the plasma membrane to the actin cytoskeleton.

Although we studied mammalian cell-culture models, we believe that PtdIns(4,5)P2 probably functions similarly in lower organisms. Genetic data implicating a PI-4-kinase in *D. melanogaster* [3] and a PI(4)P-5-kinase in *S. pombe* [1, 2] in cytokinesis can be reconciled by

observing that these form a pathway to synthesize PtdIns(4,5)P2 from PtdIns. Furthermore, data from the Brill Laboratory ([23], this issue) also suggests a requirement for PtdIns(4,5)P2 in *D. melanogaster* spermatocyte cytokinesis. However, Brill and colleagues observe diffuse localization of PtdIns(4,5)P2 without concentration at the furrow. In light of the genetic data, we would argue that PtdIns(4,5)P2 is probably performing a similar function but that in higher organisms a mechanism has evolved to enhance that function by concentrating PtdIns(4,5)P2 at the furrow. Further study will be required to test this hypothesis.

Supplemental Data

Supplemental Experimental Procedures and seven supplemental movies are available with this article online at http://www.current-biology.com/cgi/content/full/15/15/1407/DC1/.

Acknowledgments

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