

Primer Review

Regulation of the actin cytoskeleton by PIP2 in cytokinesis

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Cytokinesis is a sequential process that occurs in three phases: assembly of the cytokinetic apparatus, furrow progression and fission (abscission) of the newly formed daughter cells. The ingression of the cleavage furrow is dependent on the constriction of an equatorial actomyosin ring in many cell types. Recent studies have demonstrated that this structure is highly dynamic and undergoes active polymerization and depolymerization throughout the furrowing process. Despite much progress in the identification of contractile ring components, little is known regarding the mechanism of its assembly and structural rearrangements. PIP2 (phosphatidylinositol 4,5-bisphosphate) is a critical regulator of actin dynamics and plays an essential role in cell motility and adhesion. Recent studies have indicated that an elevation of PIP2 at the cleavage furrow is a critical event for furrow stability. In this review we discuss the role of PIP2-mediated signalling in the structural maintenance of the contractile ring and furrow progression. In addition, we address the role of other phosphoinositides, PI(4)P (phosphatidylinositol 4-phosphate) and PIP3 (phosphatidylinositol 3,4,5-triphosphate) in these processes.

Overview: regulation of the actin cytoskeleton by PIPKs (phosphatidylinositol phosphate kinases) and PIP2 (phosphatidylinositol 4,5-bisphosphate)

PIP2 is generated by the activity of type I (PIPKI) or type II (PIPKII) kinase isoforms (α , β , γ) which utilize PI(4)P (phosphatidylinositol 4-phosphate) and PI(5)P (phosphatidylinositol 5-phosphate) as substrates respectively. PIPKs are localized to the plasma membrane and are thought to account for the majority of PIP2 synthesis, whereas PIPKII

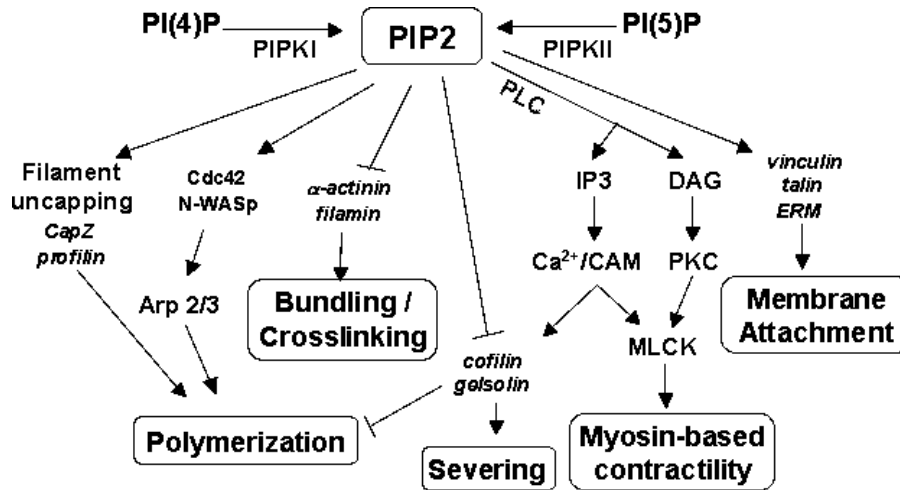
are predominantly localized to intracellular sites (Doughman et al., 2003). PIP2 plays a key role in re-structuring the actin cytoskeleton in several ways. In general, high levels of PIP2 are associated with actin polymerization, whereas low levels block assembly or promote actin severing activity. PIP2 facilitates actin polymerization in multiple ways such as: (i) activating N-WASp (neuronal Wiskott–Aldrich syndrome protein)- and Arp2/3 (actin-related protein 2/3)-mediated actin branching, (ii) binding and impairing the activity of actin-severing proteins, such as gelsolin and cofilin/ADF (actin depolymerizing factor); and (iii) uncapping actin filaments for the addition on new actin monomers (Sechi and Wehland, 2000; Yin and Janmey, 2003). This polymerization signal is counteracted by the generation of IP3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol), following PLC (phospholipase C)-mediated hydrolysis of PIP2. IP3-mediated activation of Ca^{2+} /CaM (calmodulin) promotes the activation of severing proteins such as gelsolins (McGough et al., 2003) and cofilin (Meberg, 2000), which lead to solubilization of the actin network (Figure 1). In addition to influencing actin polymerization, PIP2 modulates the function of several actin cross-linking and regulatory proteins which are critical for the assembly of stress fibres, gel meshworks and membrane

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Abbreviations used: ARAP3, Arp GAP and Rho GAP with ankyrin repeat and PH domains protein 3; ARF6, ADP-ribosylation factor 6; CaM, calmodulin; CHO, Chinese-hamster ovary; DAG, diacylglycerol; ERM, ezrin/radixin/moesin; GAP, GTPase-activating protein; GEF, guanine nucleotide-exchange factor; GFP, green fluorescent protein; IP3, inositol 1,4,5-triphosphate; IQGAPs, IQ motif-containing GTPase-activating protein; MARCK, myristoylated alanine-rich C kinase substrate; MgcRacGAP, male-germ-cell Rac GTPase-activating protein gene; MLC, myosin regulatory light chain; MLCK, MLC kinase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PI4K, phosphoinositide 4-kinase; PIP, phosphatidylinositol phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(5)P, phosphatidylinositol 5-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PIPK, PIP kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; ROCK, Rho kinase.

Figure 1 | Summary of PIP2-mediated regulation of the actin cytoskeleton



attachment. For example, PIP2 negatively regulates cross-linking mediated by filamin (Furuhashi et al., 1992) and the actin-bundling activity of α -actinin (Fraleley et al., 2003). In contrast, PIP2 induces conformational changes in vinculin, talin and ERM (ezrin/radixin/moesin) family proteins to promote anchoring of the actin cytoskeleton to the plasma membrane (Sechi and Wehland, 2000). PLC-mediated hydrolysis of PIP2 and the downstream activation of Ca^{2+} /CaM and PKC (protein kinase C) also influences actin-myosin based contractility. Ca^{2+} /CaM activates MLCK (myosin regulatory light chain kinase), leading to phosphorylation of the MLC (myosin regulatory light chain) (Iwasaki et al., 2001). Similarly, PKC has been shown to phosphorylate and activate MLC (Naka et al., 1988; Varlamova et al., 2001) (Figure 1).

Role of PIP2-mediated signalling in cell division

Prior to cell division cells undergo a global cell rounding which is a prerequisite step for the initiation of the cleavage furrow. In frog, sea urchin and newt eggs these shape changes correlate with an increase in cortical tension that precedes or occurs near the onset of the cleavage furrow (Sawai and Yoneda, 1974; Yoneda et al., 1982; Bement and Capco, 1990). Precise mapping of the changes in cortical tension have shown that peaks of tension are propagated in waves that occur in front of and at the same time

as furrow initiation. These tension waves are generated by actomyosin-based contractility and subside after the furrow has passed (Sawai and Yoneda, 1974; Yoneda et al., 1982; Christensen et al., 1984). Experiments in *Xenopus* eggs, zebrafish and *Xenopus* embryos indicated that site-specific Ca^{2+} waves were generated within the cleavage furrow that would be predicted to coincide with peaks of cortical tension. The injection of heparin, a competitive inhibitor of IP3 receptors, or Ca^{2+} chelators were both demonstrated to significantly delay or arrest furrowing (Han et al., 1992; Miller et al., 1993; Lee et al., 2003). Han et al. (1992) observed a similar inhibitory effect of microinjected PIP2 antibodies that caused a depletion of the intracellular pool of DAG and Ca^{2+} in *Xenopus* blastomeres. In addition, the increase in cortical contractility of *Xenopus* oocytes has been shown to occur via a PKC-dependent pathway (Capco et al., 1992). Together, these studies demonstrate a role for PIP2-mediated signalling at the early stages of cytokinesis.

Recent studies have supported that PIP2-mediated signalling also plays a critical role in ingression of the cleavage furrow, although significant differences have been shown in the localization of PIP2 and the role of PLC. Saul et al. (2004) reported that lithium and the PLC inhibitor, U73122, caused a rapid (within minutes) regression of cleavage furrows in crane fly spermatocytes, but did not block their initial formation. PIP2 levels were not examined in the study carried out by Saul et al. (2004), nor was the site

of PIP2 hydrolysis identified. However, a follow-up study in *Drosophila* spermatocytes demonstrated uniform distribution of GFP (green fluorescent protein)-labelled PLC δ PH (pleckstrin homology) domain in the plasma membrane, which did not appreciably increase in intensity during cell division (Wong et al., 2005). Furrow regression of *Drosophila* spermatocytes was demonstrated using U73122, the Ca²⁺ chelator, BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)], and a PIP2-specific sequestering peptide, PBP10. Interestingly, the impaired phenotype of U73122-treated cells was rescued, although not in all cells, by the addition of the Ca²⁺ ionophores, A23187 or ionomycin. These observations suggest that generation of the Ca²⁺ signal is critical for furrow stability. However, it was noted that both thapsigargin (which releases intracellular endoplasmic reticulum Ca²⁺) or phorbol ester (an activator of PKC) did not cause a similar rescue of U73122-treated cells. Furthermore, the relevance of these findings for cells *in vivo* is uncertain, since the concentration of Ca²⁺ used was significantly higher than physiological levels.

Other data suggest that PIP2 may become concentrated within the cleavage furrow and could facilitate anchoring of the plasma membrane to structural components of the actomyosin ring. A PIPKI homologue, *its3*, and PIP2 were reported at the septum of dividing fission yeast, *Schizosaccharomyces pombe*. A temperature-sensitive mutant of *its3* exhibited disrupted actin patches, following a shift to the restrictive temperature, and also impaired cytokinesis. Although a contractile ring was still evident in these cells, abnormalities, such as an extra ring, were found (Zhang et al., 2000). Two recent studies by Emoto et al. (2005) and Field et al. (2005b) demonstrated an increase in PIP2-specific GFP-labelled PH domains within the cleavage furrow of mammalian cells. In both of these reports, the intensity of the GFP signal did not appear to correlate with increased membrane surface area, as indicated by staining with membrane markers, which suggested *de novo* synthesis of PIP2 occurs within the furrow. In support of this notion, Emoto et al. (2005) found that endogenous and over-expressed PIPKI β , but not PIPKI γ , concentrated in the cleavage furrow of CHO (Chinese-hamster ovary) cells. The expression of a kinase-dead mutant of this isoform and microinjection of PIP2-specific antibodies both caused a significant increase

in the number of multinucleated cells (Emoto et al., 2005). A multinucleated phenotype was, similarly, observed by Field et al. (2005b) in multiple cell lines (CHO, HeLa, NIH 3T3 and 293T) transfected with high levels of PIP2-specific PH domains, synaptojanin [which dephosphorylates PIP2 to PI(4)P], or a kinase-dead mutant of PIPKI α . In addition, a small percentage of CHO and HeLa cells expressing high levels of PIP2-specific PH domains or synaptojanin showed signs of F-actin dissociation from the plasma membrane. However, the abundance of F-actin within the furrow was not appreciably different from controls, suggesting polymerization and/or recruitment of F-actin was not significantly altered. CHO cells transfected with PIP2 PH domains, but not PH domains specific for PI(3,4)P2 (phosphatidylinositol 3,4-bisphosphate) and PIP3, were also shown to exhibit impaired furrow expansion induced by the application of hypotonic buffer. This suggests one of the primary roles of PIP2 is to promote cytoskeleton–membrane anchoring at the furrow.

Role of PI3Ks (phosphoinositide 3-kinases) and PI4Ks (phosphoinositide 4-kinases) in cytokinesis

PI4Ks generate the PIPKI substrate, PI(4)P, and play a critical role in PIP2 generation. Although not as well studied as PIPKs, studies in lower organisms support the requirement of PI4Ks for cytokinesis. In *Saccharomyces cerevisiae* two PI4Ks, *STT4* and *PIK1*, have non-overlapping functions in Golgi-to-membrane trafficking and cell-wall integrity respectively (Hama et al., 1999; Audhya et al., 2000). Both genes are also required for cell division. Conditional mutants of *Pik1p* exhibited a cytokinesis defect: cells arrest with large buds and fully divided nuclei (Garcia-Bustos et al., 1994). In addition, *STT4* was identified as a gene implicated in reorientation of the mitotic spindle prior to cytokinesis. However, the precise role of *STT4* in this process has not been characterized (Muhua et al., 1998). Brill et al. (2000) identified a *Drosophila* PI4K, *fwd* (*four wheel drive*), as a gene required for cytokinesis in male germ cells. Spermatocytes derived from *fwd* mutant males had unstable furrows that failed to ingress and abnormal contractile rings with dissociated myosin II and F-actin (Brill et al., 2000). *fwd* has homology

with yeast *PIK1* and human PI4KIII β . Although *PIK1* is an essential gene in yeast, the deletion of *fwd* was not lethal and female flies were fertile. It was suggested by Brill et al. (2000) that the cytokinesis defect in *fwd* mutant spermatocytes may be due to a specific role of *fwd* in these cells, or that they may be deficient in the expression of other PI4Ks. Although it was not determined if *fwd* was recruited to the cleavage furrow, a study in fission yeast suggests that PI4Ks may be recruited to the furrow, as reported for PIPKs. Desautels et al. (2001) identified a PI4K as a binding partner of Cdc4p, a contractile ring protein with homology to the myosin essential light chain. A Cdc4p mutant, G107S, abolished the interaction with PI4K and induced the formation of multinucleated cells with defects in septum formation (Desautels et al., 2001). This finding suggests that, at least for fission yeast, anchoring of PI4K to the contractile ring may concentrate PI(4)P substrate within the furrow for subsequent PIP2 generation.

In mammalian cells, little is known about the role of PI4K isoforms during cell division. Mammalian homologues of Pik1p and Stt4p are classified as type III PI4Ks (PI4KIII α and PI4KIII β) and, similar to PI3Ks, are wortmannin-sensitive. Another class of PI4Ks, the type II enzymes (PI4KII α and PI4KII β), are wortmannin-insensitive. In contrast with PIPKs, which predominantly generate PIP2 at the plasma membrane, mammalian PI4Ks are largely localized to intracellular regions, such as the Golgi (PI4KIII β , PI4KII α and PI4KII β) and the endoplasmic reticulum (PI4KIII α) (Wong et al., 1997; Wei et al., 2002; Weixel et al., 2005). This suggests that PI(4)P and PIP2 synthesis may be uncoupled in mammalian cells. However, recruitment of PI4KII β and PI4KIII α to the plasma membrane has been reported (Wei et al., 2002; Balla et al., 2005). Field et al. (2005b) reported that, in contrast with PIP2, PI(4)P did not accumulate at the cleavage furrow of NIH 3T3 cells. This could indicate PI4Ks are not active at this site. Alternatively, it is possible that the increased PIP2 synthesis by PIPKs may account for the depletion of PI(4)P within the furrow. Further studies are needed to determine if specific PI4K isoforms are required for cytokinesis of mammalian cells.

In contrast with PIPKs and PI4Ks, there is little evidence that PI3Ks are required at the time of fur-

row initiation. Although few studies have examined PI3K function at this stage of the cell cycle, current experimental evidence suggests that PI3K activity may need to be suppressed for proper furrow development. PI3Ks are composed of a regulatory subunit (p85) and a catalytic subunit (p110), and are functionally grouped into two classes. Class IA PI3Ks (PI3K α , PI3K β , PI3K δ) are activated via tyrosine kinase receptors and cytosolic tyrosine kinases, whereas class IB PI3Ks (PI3K γ) are activated by heterotrimeric G-protein $\beta\gamma$ subunits. PI3K-mediated activation of the Akt/protein kinase B pathway is a critical event for several essential cellular processes, such as cell migration, growth and cell-cycle entry (Vanhaesebroeck and Alessi, 2000). This signalling cascade is dependent on the generation of PIP3 from PIP2 by PI3Ks and balanced by phosphatases, such as PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIP-1/2 (SH2-containing inositol phosphatase 1/2) that dephosphorylate PIP3 to PIP2 and PI(3,4)P2 respectively (Leslie and Downes, 2004).

In crane fly spermatocytes, PI3K inhibitors, wortmannin and LY294002, were shown to impair cytokinesis in a similar manner to pharmacological inhibition of PLC by U73122 (Saul et al., 2004). The significance of this finding, however, is difficult to interpret, since PIP3 levels were not examined in the study by Saul et al. (2004), and both wortmannin and LY294002 have the potential to impair PI4K. In addition, other studies have indicated PIP3 was not present during furrow initiation in *Dictyostelium discoïdum*, *Drosophila* spermatocytes or mammalian cells (Emoto et al., 2005; Field et al., 2005b; Janetopoulos et al., 2005; Wong et al., 2005). Furthermore, the PIP3 phosphatase, PTEN, has been reported at the septum of dividing fission yeast (Mitra et al., 2004) and cleavage furrow of *Dictyostelium* (Janetopoulos et al., 2005). In *Dictyostelium*, PIP3 was detected at a late stage of cytokinesis after cells had elongated and was localized to the pole regions (Janetopoulos et al., 2005). Janetopoulos et al. (2005) hypothesized that activation of PI3K at the poles may play a role in actin polymerization and membrane expansion for the completion of cytokinesis. At the cleavage furrow this signal is repressed by PTEN, since it would presumably interfere with furrow ingression. It remains to be determined if the orientation of PI3K and PTEN to the poles and furrow

respectively is found in other cell types. This model may be applicable to other cells, however, as PIP3 is functionally required for actin polymerization and membrane protrusion at leading edges of migrating cells (Merlot and Firtel, 2003). Interestingly, the recruitment of PTEN to the plasma membrane is dependent on its interaction with PIP2 via its PH domain (Campbell et al., 2003). This raises an interesting possibility that elevated PIP2 within the furrow may serve to recruit PTEN to this site. Although PIP2 was not examined at the furrow of *Dicystostelium*, PIP2 was elevated at the septum of fission yeast (Zhang et al., 2000).

Structural maintenance of the contractile ring: sustained PIP2, hydrolysis or both?

The ingression of the cleavage furrow and successful segregation of daughter cells is critically dependent on the constriction of an equatorial actomyosin ring in several cell types (Balasubramanian et al., 2004). In contrast with previous models, which predicted the ring to be a stable structure, accumulating evidence supports the idea that the contractile ring is highly dynamic. For example, the ring components actin, myosin and tropomyosin are rapidly turned over each minute in dividing fission yeast (Pelham and Chang, 2002). Similar evidence for cycles of assembly and disassembly of ring components has been demonstrated in vertebrate cells (Savoian et al., 1999). Actin-binding proteins localized to the furrow, such as anillin, septins and ERM proteins, are thought to regulate the organization of the medial ring and/or anchoring to the plasma membrane (Sato et al., 1991; Kinoshita et al., 2002; Straight et al., 2005; Yokoyama et al., 2005; Zhao and Fang, 2005a). In addition, proteins that regulate actin filament dynamics, such as profilin, diaphanous-related formins (Dia) and cofilin, are required for cytokinesis (Castrillon and Wasserman, 1994; Giansanti et al., 1998; Somma et al., 2002). A common feature of these protein families is that phosphoinositides modulate their activity and/or structure.

An increased synthesis of PIP2 by PIPKs at the cleavage furrow is anticipated to promote both actin polymerization and structural support to the contractile ring. In the resting state, the barbed (fast-growing) ends of actin filaments are occupied by

capping proteins. In the absence of free barbed ends, the actin-nucleating protein, profilin, binds and sequesters free actin monomers. This helps to prevent spontaneous elongation of actin filaments. The up-regulation of PIP2 promotes uncapping of actin filaments and impairs the filament severing activity of cofilin. Profilin-actin complexes can then be added to the free ends of actin filaments (dos Remedios et al., 2003; Yin and Janmey, 2003). This process is facilitated by activated formins, which utilize profilin-actin complexes as substrates for actin nucleation (Watanabe and Higashida, 2004). It has been suggested that incorporation of actin into the growing filament requires the dissociation of profilin from the barbed end (Nyman et al., 2002). Phosphoinositides, such as PIP2, PIP3 and PI(3,4)P2, may facilitate this process, since they each have been shown to disrupt profilin-actin complexes (Lu et al., 1996; Skare and Karlsson, 2002). Thus an increase in PIP2 synthesis within the cleavage furrow could enhance the rate of profilin-actin disassembly, and thereby enhance actin polymerization.

Structural proteins of the contractile ring regulated by PIP2 include anillin, septin and ERM proteins. The concentration of PIP2 at the cleavage furrow is postulated to be a critical molecule in the recruitment of these proteins and their integration with the actomyosin ring. Anillin exhibits actin-bundling activity and is required at the terminal stages of cytokinesis in *Drosophila* and human cells (Somma et al., 2002; Straight et al., 2005; Zhao and Fang, 2005a). The depletion of anillin in *Drosophila* and human cells causes cytokinesis failure, which is correlated with uncoordinated actomyosin contraction of the medial ring (Straight et al., 2005). Anillin also functions as a cofactor to promote the recruitment of septins to actin bundles (Kinoshita et al., 2002). Mutations within the PH domain of anillin were recently demonstrated to impair septin localization to both the furrow canal and the contractile ring in *Drosophila* cells, blocking cellularization and furrow progression (Field et al., 2005a). Septins have also been shown to bind to phosphoinositides and this interaction regulates their subcellular localization. The mammalian septin, H5, bound PIP2 and PIP3 liposomes at its N-terminal basic region, which is conserved in most septin proteins. The over-expression of synaptojanin and treatment with neomycin (which depletes cellular PIP2) both caused disruption of actin stress

fibres and dissociation of H5 from filamentous structures in Swiss 3T3 cells (Zhang et al., 1999). In addition, a budding yeast septin, Cdc11, was reported to interact with PI(4)P and PI(5)P (Casamayor and Snyder, 2003). Septins are co-localized with actin at the cleavage furrow and form ring structures that are postulated to structurally support the contractile ring (Kinoshita et al., 2002). ERM proteins are also concentrated within the cleavage furrow and provide a structural link between F-actin and the plasma membrane (Sato et al., 1991; Kosako et al., 2000; Yokoyama et al., 2005). Although the precise mechanism is not fully understood, both binding of PIP2 to the conserved FERM (four-point-one/ERM) domain and threonine phosphorylation of the F-actin domain have been implicated in ERM activation. Phosphorylation of ERM proteins is mediated by the RhoA effector ROCK (Rho kinase) and PKCs (Bretscher et al., 2002). It has been suggested that interaction with PIP2 is a prerequisite step for subsequent phosphorylation of the ERM activating residue Thr-567 (Fievet et al., 2004).

Studies by Saul et al. (2004) and Wong et al. (2005) suggest that PLC-mediated hydrolysis of PIP2 and the subsequent release of intracellular Ca^{2+} stores is a necessary event for furrow stability and ingression. A role for Ca^{2+} is similarly supported by previous findings that Ca^{2+} waves were localized to the cleavage furrow in frog embryos, eggs and fish embryos (Han et al., 1992; Miller et al., 1993; Lee et al., 2003). PLC second messengers have also been implicated in cytokinesis. For example, CaM was localized to mitotic spindles of HeLa cells and the inhibition of its activity was reported to cause cytokinesis defects (Yu et al., 2004). In addition, PKCs have been functionally implicated in MLC phosphorylation during mitosis and assembly of the mitotic spindle (Varlamova et al., 2001; Chen et al., 2004). Despite this evidence, it is presently unclear if PLC is recruited to the cleavage furrow and if the Ca^{2+} signal occurs before or after the furrow commences. For example, Noguchi and Mabuchi (2002) showed that Ca^{2+} waves formed at a late stage of cleavage in *Xenopus* eggs and injection of dibromo-BAPTA or EGTA at a level that was sufficient to suppress these waves did not block furrowing. A recent RNAi (RNA interference) screen also identified PI4Ks and PIPKs, but not PLC genes, as critical proteins for cytokinesis in *Drosophila* (Eggert et al., 2004). This may indicate that

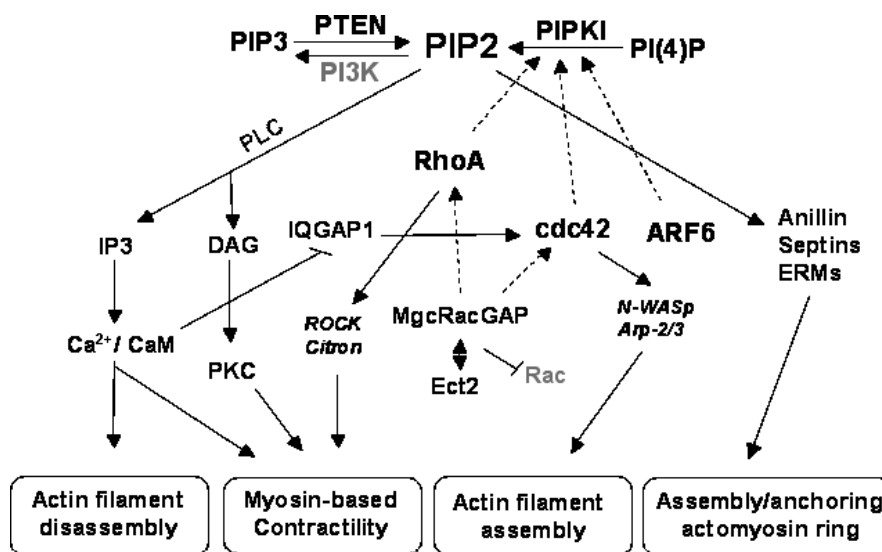
PLC is required for completion of furrowing, rather than its initiation.

It is hypothesized that PLC activity may promote actin filament severing through the activation of Ca^{2+} -dependent actin-severing proteins, such as gelsolin and cofilin (Yin and Janmey, 2003). Depending on the localization of PLC, this could either drive disassembly of actin filaments of the medial ring or the cortical actin network. Furthermore, the activation of PKC and CaM would activate actomyosin contraction via the phosphorylation of MLCK. At the furrow, PKC and CaM could act in concert with the Rho effectors ROCK and Citron kinase, which also phosphorylate and activate MLC (Kosako et al., 2000; Yamashiro et al., 2003) (Figure 2). Saul et al. (2004) have suggested that PLC activity may be required for the migration of cortical actin filaments into the cleavage furrow. They observed that crane fly spermatocytes treated with U73122 exhibited a depletion of actin within the furrow and an accumulation at the pole regions. Although the site of PIP2 hydrolysis was not identified, the authors proposed that PLC activity at the poles could promote actin filament severing, which may be required to free them for migration into the furrow (Saul et al., 2004). This hypothesis is consistent with previous studies that have shown the cortical flow of pre-existing actin filaments into the furrow (Cao and Wang, 1990) and actin polymerization at the pole regions of mammalian cells (O'Connell et al., 2001). Furthermore, it has been shown that the cortical flow of actin filaments to wound-induced contractile rings of *Xenopus* oocytes is dependent on actomyosin contractility (Mandato and Bement, 2001). Thus it is plausible that activation of PLC could provide the necessary signals to release cortical actin filaments and drive their movement.

The activation of CaM and/or PKC may also provide positive feedback for the recruitment of PIP2 effectors and regulate GTPase-mediated actin polymerization. Both PKC and CaM have been shown to promote the dissociation of MARCKS (myristoylated alanine-rich C kinase substrates) family proteins from PIP2. MARCKS are postulated to play a major regulatory role in phosphoinositide signalling by sequestering PIP2 at the membrane. Thus the activation of PKC and CaM promotes PIP2 availability for the recruitment of PH-domain-containing effector proteins (Lanier and Gertler, 2000). In addition,

Figure 2 | Proposed model of PIP2 and GTPase signalling at the cleavage furrow

Ect2, is recruited to the cleavage furrow via its interaction with MgcRacGAP at the central spindle. Ect2 and MgcRacGAP regulate the activities of Rho GTPases (RhoA, Cdc42 and Rac) and are functionally implicated in the assembly of the contractile ring. Active RhoA and Cdc42 are increased at the furrow, whereas Rac is suppressed (grey). Furrow-recruited GTPases (RhoA, ARF6 and Cdc42) are predicted to activate PIPKI, leading to the generation of PIP2. PI3K activity is suppressed at the furrow (grey), which may be due to MgcRacGAP-mediated inhibition of Rac and/or the activity of the PIP3 phosphatase, PTEN. Cycles of PIP2 synthesis and hydrolysis by PLC are thought to play a critical role in re-structuring the contractile ring throughout the duration of furrowing. PIP2-mediated activation of anillin, septins and ERM proteins promotes cross-linking and membrane anchoring of the contractile ring. PLC-mediated activation of PKC and CaM can facilitate the contraction of the actomyosin ring, similar to RhoA effectors, ROCK and Citron kinase. CaM may also regulate IQGAP–Cdc42 interactions, and thereby modulate actin organization. It is hypothesized that Cdc42-mediated actin polymerization via effectors, such as N-WASp (neuronal Wiskott–Aldrich syndrome protein) and Arp2/3 (actin-related protein 2/3), may reduce membrane tension outside the inner region of RhoA-mediated contractility.



studies in yeast and mammalian cells have supported that CaM and PKC can mediate positive feedback for PIP2 synthesis by activating PIPKs (Park et al., 2001; Desrivieres et al., 2002). CaM also regulates actin filament organization via its interaction with IQGAPs (IQ motif-containing GTPase-activating proteins). IQGAPs are GTPase-stabilizing proteins that selectively bind Rac and Cdc42 over RhoA, and also directly interact with F-actin. In fission and budding yeast, IQGAPs are required for the assembly of the actomyosin ring (Eng et al., 1998; Shannon and Li, 1999). In mammalian cells, CaM was demonstrated to impair the formation of IQGAP1–Cdc42 complexes that promote F-actin cross-linking (Mateer et al., 2002). This further strengthens the notion that PLC-mediated signalling may be required for remodelling of the contractile ring as the cleavage furrow progresses.

Signalling crosstalk: role of GTPases and phosphoinositides

On the basis of the present available data, PIP2 has been shown to be a critical molecule for structural integrity of the contractile ring and furrow stability. However, the observation that furrows are initiated in cells treated with agents that either sequester PIP2 or prevent its hydrolysis suggests PIP2 does not provide the originating signal for furrow formation. Recent studies suggest that the recruitment and activation of RhoA may provide this early signal. Bement et al. (2005) found that active RhoA was concentrated to the equatorial region of sea urchin and *Xenopus* embryos prior to the onset of cytokinesis, and that this localization was perturbed by micro-manipulation of the spindle and nocodazole treatment. Studies in HeLa cells demonstrated that the Rho GEF (guanine nucleotide-exchange factor), Ect2,

which is required for cytokinesis and Rho activity, interacts with the central spindle component, MgcRacGAP (male-germ-cell Rac GTPase-activating protein gene) (Kamijo et al., 2005; Yuce et al., 2005; Zhao and Fang, 2005b). Moreover, the depletion of MgcRacGAP was shown to block furrow initiation and caused impaired recruitment of Ect2, anillin and myosin II (Yuce et al., 2005; Zhao and Fang, 2005b). These studies demonstrate that the activation of RhoA plays an important role in contractile ring assembly and furrow initiation.

In addition to regulating RhoA, Ect2 and MgcRacGAP also play a critical role in regulating the activities of Cdc42 and Rac during cytokinesis. In two separate studies, Yoshizaki et al. (2003, 2004) reported that MgcRacGAP activity was required for the suppression of Rac activity at the onset of cytokinesis in HeLa and Rat1A cells. The down-regulation of Rac was critical for proper cell division, as expression of a constitutively active Rac mutant caused a multinucleated phenotype (Yoshizaki et al., 2003, 2004). In contrast to Rac, Cdc42 appears to be required at several stages of cytokinesis. Ocegüera-Yanez et al. (2005) observed that GTP-bound Cdc42, but not Rac, was up-regulated during metaphase and recruited to the central spindle of HeLa cells. Cdc42 activity was impaired by the over-expression of dominant negative forms of Ect2 and MgcRacGAP, and resulted in impaired chromosome segregation. A similar impaired phenotype was observed in Cdc42-depleted cells. Other observations have provided evidence that Cdc42 is required for the septin organization in fission yeast (Caviston et al., 2003) and spindle assembly in *Xenopus* egg extracts (Tatsumoto et al., 2003). It has not been determined if RhoA and Cdc42 are localized to distinct sites at the cleavage furrow or how these signals may co-ordinate the furrowing process. However, a recent study by Bement et al. (2005) suggests the potential for spatially defined zones of these GTPases. They observed that active RhoA and Cdc42 formed discrete inner and outer rings respectively at the border of wound-induced contractile arrays of *Xenopus* oocytes. These zones were maintained throughout the duration of wound closure, which is driven by constriction of an actomyosin ring, similar to cytokinesis (Benink and Bement, 2005). The authors postulated that Cdc42-mediated actin polymerization in the outer ring could promote membrane expansion and define a region of

low tension. This would counterbalance the high tension of the inner ring that undergoes RhoA-mediated actomyosin contraction. It remains to be determined if such an arrangement occurs at the cleavage furrow of dividing cells.

It is anticipated that the activity of furrow-recruited GTPases plays a critical role in the recruitment and activation of phosphatidylinositol kinases during cytokinesis. In addition to RhoA, the GTPase ARF6 (ADP-ribosylation factor 6) was recruited to the cleavage furrow and its activity required for cytokinesis in HeLa cells (Schweitzer and D'Souza-Schorey, 2002). The activation of RhoA and ROCK can mediate the recruitment and activation of PIPKs (Chong et al., 1994; Oude Weernink et al., 2000). Similarly, ARF6 can activate PIPK α in the presence of phosphatidic acid generated by PLD (phospholipase D) (Honda et al., 1999). It has not been determined if PLD is recruited to the cleavage furrow, similar to ARF6. However, the lipid environment of the cleavage furrow suggests this is plausible. Both mammalian PLD enzymes, PLD1 and PLD2, have a strict requirement for PIP2 for their activation (Liscovitch et al., 1994; Hurley and Meyer, 2001), which appears to be increased at the furrow. In addition, Emoto and Umeda (2000) have shown that PE (phosphatidylethanolamine), an activating cofactor of PLD (Nakamura et al., 1996), is concentrated within the furrow of dividing CHO cells. Together, these findings suggest that PLD and ARF6 may be important components of a positive feedback cycle for PIP2 synthesis.

GTP-bound forms of Rac1 and Cdc42 have both been shown to bind regulatory and catalytic subunits of PI3K, and this interaction enhanced PI3K activity (Zheng et al., 1994; Kobayashi et al., 1998). It has been suggested, however, that Rac could be a predominant activator of PI3K (Srinivasan et al., 2003), which is supported by the finding that Rac and PIP3 are both localized at the leading edge of migrating cells (Kraynov et al., 2000; Merlot and Firtel, 2003). Therefore, it is possible that the decline in Rac activity at the onset of cytokinesis is, in part, responsible for the low level of PIP3 that is similarly observed (Emoto et al., 2005; Field et al., 2005b; Wong et al., 2005). In turn, it can be hypothesized that the selective generation of PIP2 over PIP3 may play a role in providing appropriate signalling feedback to furrow-recruited GTPases. For example, PIP3 has been shown to activate the Rac GEF, Vav,

whereas PIP2 was found to be inhibitory (Das et al., 2000). The ARF6 GAPs (GTPase-activating proteins), ARAP3 (Arf GAP and Rho GAP with ankyrin repeat and PH domains protein 3) and centaurin- α 1, which down-regulate ARF6, are also recruited by PIP3. ARAP3 exhibits selective GAP activity for RhoA over Cdc42 and Rac (Krugmann et al., 2004; Venkateswarlu et al., 2004). This suggests the generation of PIP3 has the potential to impair furrowing by activating Rac and/or inhibiting RhoA and ARF6. In the case of Ect2, it is uncertain if phosphoinositides play a significant role in determining its localization and activity. Similar to many other Dbl family GEFs, Ect2 contains a DH (Dbl homology) domain, followed by a PH homology domain in its C-terminal region. However, phosphoinositide binding is not required for activation of all members of this family (Rossman et al., 2005). Tatsumoto et al. (1999) found that a truncated form of Ect2, lacking the PH domain and C-terminus, localized to the midbody of HeLa cells, similar to endogenous Ect2, and impaired cell division. However, a study by Solski et al. (2004) demonstrated that a mutation in the PH domain of Ect2 did not impair its activity. These observations suggest that interaction with phosphoinositides may not be essential for the recruitment and activation of Ect2 within the furrow. This notion appears to be consistent with the reported roles of the MgcRacGAP–Ect2 complex and PIP2 in cytokinesis. Whereas MgcRacGAP depletion impaired Ect2 recruitment, contractile ring assembly and furrow initiation (Yuce et al., 2005; Zhao and Fang, 2005b), the impairment of PIP2 signalling by sequestering peptides or the PLC inhibitor, U73122, were shown to block furrow stability, but not its initiation (Saul et al., 2004; Field et al., 2005b; Wong et al., 2005). Thus present evidence would suggest Ect2 is activated prior to the increase in PIP2 synthesis.

In conclusion, PIP2 is a critical molecule for several components of cytokinesis, including global cell rounding, cortical flow of actin and structural maintenance of the contractile ring. It is anticipated that the activity of PIPKs is dependent on furrow-associated GTPases, such as RhoA, Cdc42 and ARF6 (Figure 2). Future studies are needed to determine the precise role of specific GTPases and/or other signalling molecules that are required for the recruitment of PIPKs to the cleavage furrow. Another challenging area of research will be to further investigate the

kinetics of PIP2 synthesis and PLC-mediated hydrolysis during cytokinesis in different cell types. This may reveal conserved patterns of PIP2 cycles required for cell division. In contrast with PIP2, current data indicates that PI3K-mediated signalling and PIP3 production is suppressed during furrowing. It has been suggested that this may be required to prevent membrane expansion at the furrow which could interfere with constriction of the actomyosin ring. It is postulated that the inhibition of PI3K may be dependent on diminished Rac activity that occurs at the onset of furrowing.

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