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Phospholipase C Isoforms Are Localized at the Cleavage Furrow during Cytokinesis

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It has recently been demonstrated that phosphatidylinositol 4,5-bisphosphate (PIP_2) is localized at the cleavage furrow in dividing cells and its hydrolysis is required for complete cytokinesis, suggesting a pivotal role of PIP_2 in cytokinesis. Here, we report that at least three mammalian isoforms of phosphoinositide-specific phospholipase C (PLC), PLCδ1, PLCδ3 and PLCβ1, are localized to the cleavage furrow during cytokinesis. Targeting of the δ1 isoform to the furrow depends on the specific interaction between the PH domain and PIP_2 in the plasma membrane. The necessity of active PLC in animal cell cytokinesis was confirmed using the specific inhibitors for PIP_2 hydrolysis. These results support the model that activation of selected PLC isoforms at the cleavage furrow controls progression of cytokinesis through regulation of PIP_2 levels: induction of the cleavage furrow by a contractile ring consisting of actomyosin is regulated by PIP_2-dependent actin-binding proteins and formation of specific lipid domains required for membrane separation is affected by alterations in the lipid composition of the furrow.

Key words: actin cytoskeleton, cleavage furrow, cytokinesis, phospholipase C, PIP_2.

Abbreviations: δ1PH, the PH domain of phospholipase C-δ1; DMEM, Dulbecco's modified Eagle's medium ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; FBS, fetal bovine serum; GFP, green fluorescent protein; IP_3, inositol 1,4,5-trisphosphate; PE, phosphatidylethanolamine; PH, pleckstrin homology; PI, propidium iodide; PI(3)P, phosphatidylinositol 3-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI(4)P5K, phosphatidylinositol 4-phosphate 5-kinase; PLC, phospholipase C.

Lipids in dynamic cell membranes have two general functions: one to regulate physicochemical properties of bilayer membranes and the other to control submembranous structures, generally of cytoskeletal machineries, whose components interact with the inner surface of the plasma membrane through protein-lipid interactions or anchoring by lipid modifications of proteins. Spatio-temporal changes in the lipid composition of the membrane, therefore, serve to regulate formation of membrane domains with specific function.

Cytokinesis is a dynamic stage in mammalian cell cycle. Regulation of the actomyosin contractile ring at the cleavage furrow is important for progression of cytokinesis (*1*). To assure progression of furrowing, rapid changes in physicochemical nature of the plasma membrane itself as well as a dynamic rearrangement of cytoskeletal backbones of the membrane at the cleavage furrow may be necessary. In fact, aberrant accumulation of phosphatidylethanolamine (PE) has been reported in the outer leaflet of the plasma membrane at the cleavage furrow (*2*).

Recently, PIP_2 and its hydrolysis have gained attentions in the progression of cytokinesis (*3*, *4*). PIP_2 is not only a source of the second messenger, inositol 1,4,5-trisphosphate (IP_3), that mobilizes intracellular Ca^{2+} to control many Ca^{2+}-dependent regulators of cytokinesis, but also functions as a pivot for the cytoskeletal rearrangement *via* regulation of the actin cytoskeleton. PIP_2 plays important roles not only in actin cytoskeletal reorganization but also in vesicle trafficking, membrane ruffling and recruitment of signaling molecules (*5*). PIP_2 interacts with regulators of actin polymerization, such as profilin, cofilin, and actin capping proteins, changing their function (*5*). Generally, high levels of PIP_2 induce actin polymerization, whereas low levels of it block the polymerization or induce severing of acin filaments. PIP_2 also binds to the ERM proteins (ezrin, radixin and moesin) (*6*) and septin H5 (*7*), which are F-actin binding proteins and reported to be localized at the cleavage furrow. PIP_2 could therefore regulate the state of actin filaments at the cleavage furrow during cytokinesis (*7*, *8*).

Sequestration of PIP_2 disrupts normal cell division. Injection of mammalian cells with anti-PIP_2 antibodies results in multinucleate cells (*9*). Capping of PIP_2 with neomycin, which permeates cell membrane slowly and interfere PIP_2 binding with proteins, blocks cytokinesis in Swiss 3T3 cells (*7*). Furthermore, high expression levels of ectopic PIP_2 probes, such as the PH domain of PLCδ1 (δ1PH) and the Tubby protein's C-terminal, causes a separation of the plasma membrane from the contractile ring and increases the number of multinucleate cells due to the failure of cytokinesis (*10*).

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Thus, maintenance of the proper PIP_2 levels at the furrow seems important. For this, proper regulation of PIP_2 breakdown and PIP_2 synthesis is essential. In fission yeast, both phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5K], Its3, that binds the product, PIP_2, and the PIP_2 probe, δ1PH, localize at the septum of dividing cells (*11*). Expression of a kinase-dead mutant of PI(4)P5K reduces cytokinesis (*11*). In budding yeast, phosphoinositide-specific phospholipase C (PLC) gene, *PLC1*, which encodes a homolog of mammalian δ-type PLC, is important for cell growth (*12*). Temperature-dependent *PLC1* mutants lacking the PLC activity cease growth at random times during the cell cycle at the restrictive temperature, suggesting that active *PLC1* product is required at some or all stages of the cell cycle (*13*). In fission yeast, a mutation in the gene homologous to budding yeast *PLC1* also causes the growth defect (*14*). Furthermore, in crane fly cells, addition of U73122, the specific inhibitor for PIP_2 hydrolysis by PLC, blocks or slows down furrowing (*15*). The inhibitor also causes a regression of the cleavage furrow in a dose-dependent manner in *Drosophila* spermatocytes (*16*). These results, taken together, suggest that the presence of PIP_2 and its hydrolysis at the cleavage furrow are important in the progression of cytokinesis.

So far, thirteen mammalian PLC isoforms classified into six different subfamilies (β, γ, δ, ε, ζ, η) have been identified. Among them, the plasma membrane localization of δ-type PLC has been well documented in various cell types (*17*, *18*). Translocation of γ-type PLC to the plasma membrane has also been reported in cells stimulated with various extracellular stimuli such as epidermal growth factor and platelet-derived growth factor (*19*). The distribution of these PLC isoforms during cytokinesis, however, has not been looked at in detail.

In this study, we confirmed accumulation of PIP_2 at the cleavage furrow during cytokinesis using mammalian cell lines including epithelial MDCK cells that have not been looked at previously. We also demonstrated that hydrolysis of PIP_2 is required for progression of cytokinesis in mammalian cells using the selective PLC inhibitor, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine (ET-18-OCH_3), in addition to U73122 previously used for fly cell cytokinesis. Furthermore, we found that endogenous and ectopically expressed PLCδ1 is localized to the cleavage furrow during cytokinesis. Among several other PLC isoforms examined (two splicing variants of β1, β2, β3, γ1 and δ3), at least two isoforms, PLCβ1 and PLCδ3, are also localized to the furrow. These results suggest that there are isoform-specific roles, although some may be redundant, and targeting mechanisms for the PLC localization at the cleavage furrow during somatic cell cytokinesis.

MATERIALS AND METHODS

Plasmids—Plasmids encoding GFP-δ1PH, GFP-δ1PH R40A, GFP-PLCδ1, GFP-PLCδ1 R40A were constructed as described (*18*, *20*). Plasmids encoding GFP-PLCβ1a, GFP-PLCβ1b, GFP-PLCβ2, GFP-PLCβ3 GFP-PLCγ1 and PLCδ3 were kindly donated from Professors Pann-Ghill Suh and Song Ho Ryu (Pohang University of Science and Technology). The cDNA encoding PLCδ3 was cut out from pRSETC-PLCδ3 and subcloned into pEGFP-C1 (Clontech) using *Xho*I sites. A plasmid encoding GFP with the myristoylation signal sequence of Lyn, MGCIKSKRKD, added to the N-terminus was constructed from pEGFP-C2/PM-hPI4Kβ (a gift of Dr. Andreas Jeromin, Mount Sinai Hospital) by removing the inserted sequence for human phosphatidylinositol 4-kinase-β with *Sal*I treatment.

Cell Culture and Transfection—Cells were maintained in Dulbecco's modified Eagle's medium (DMEM: Nissui) supplemented with 10% (for HeLa and NIH-3T3 cells) or 5% (for MDCK cells) fetal bovine serum (FBS), L-glutamine and antibiotics at 37°C in 5% CO_2-humidified atmosphere. HeLa cells stably expressing GFP-PLCδ1 were established as described for MDCK cells stably expressing GFP-PLCδ1 (*18*). HeLa cells were transfected with Lipofectin (Invitrogen) and NIH-3T3 cells with Fugene-6 (Roche) according to the manufacturer's instruction.

Fluorescence Microscopy of Synchronized Cells—Cells (1×10^5) were seeded on poly-L-lysine coated coverslips in 35 mm dishes. After overnight growth, cells were transfected with 1 μg plasmid with transfection reagents as above. Twenty four hours after transfection, cells were incubated with 100 ng/ml nocodazole for 8–11 h, washed three times and incubated with fresh growth medium at 37°C for 45 min. NIH3T3 cells were fixed with 3% formaldehyde in PBS at room temperature for 10 min. MDCK and HeLa cells were methanol fixed. The cells were then permeabilized and blocked by 0.1% Triton X-100, 2% FBS at room temperature for 10 min, washed and treated with anti-PLCδ1 antibodies; a polyclonal antibody raised against rat PLCδ1 for NIH3T3 cells, a monoclonal antibody (BD Transduction Lab) for HeLa and MDCK cells, followed by staining with FITC-labeled anti-rabbit or anti-mouse IgG. Cell nuclei were visualized by staining with propidium iodide (PI: Sigma) and F-actin with Alexa-594 phalloidin (Molecular Probes). Fluorescent images were viewed on a Zeiss Axioplan 2 microscope equipped with a confocal laser scanning unit (Carl Zeiss LSM510).

Time-Lapse Fluorescence Microscopy—HeLa cells or MDCK cells stably expressing GFP-PLCδ1 were seeded on 35 mm glass bottom dishes (Mat-Teck No.1.5). Cells were treated with Hoechst-33342 (200 ng/ml in the growth medium) at 37°C, in 5% CO_2-humidified atmosphere for 20 min and washed three times with the observation medium: phenol red-free DMEM supplemented with 10% FBS, 25 mM Hepes (pH7.5), 80 μg/ml kanamycin. GFP and Hoechst images were taken by fluorescence microscopy (Olympus IX71) every 10 min at 37°C.

Paired Cell Counting—Cells (2×10^4) were seeded on 35 mm dish and incubate at 37°C for 48 h. Cells were treated with a PLC inhibitor, U73122 (Upjohn) or ET-18-OCH_3 (Calbiochem), for 6 h and paired cells in random fields were counted by phase-contrast microscopy (Nikon TMS).

Immunoblot Analysis—Cell lysates were mixed with Laemmili sample buffer, separated by SDS-PAGE and subjected to immunoblotting analysis with a monoclonal antibody against GFP (Clontech).

RESULTS AND DISCUSSION

The PH domain of PLCδ1 (δ1PH) has a high affinity for PIP_2 but its mutant, δ1PH R40A, does not (*17*, *21*).

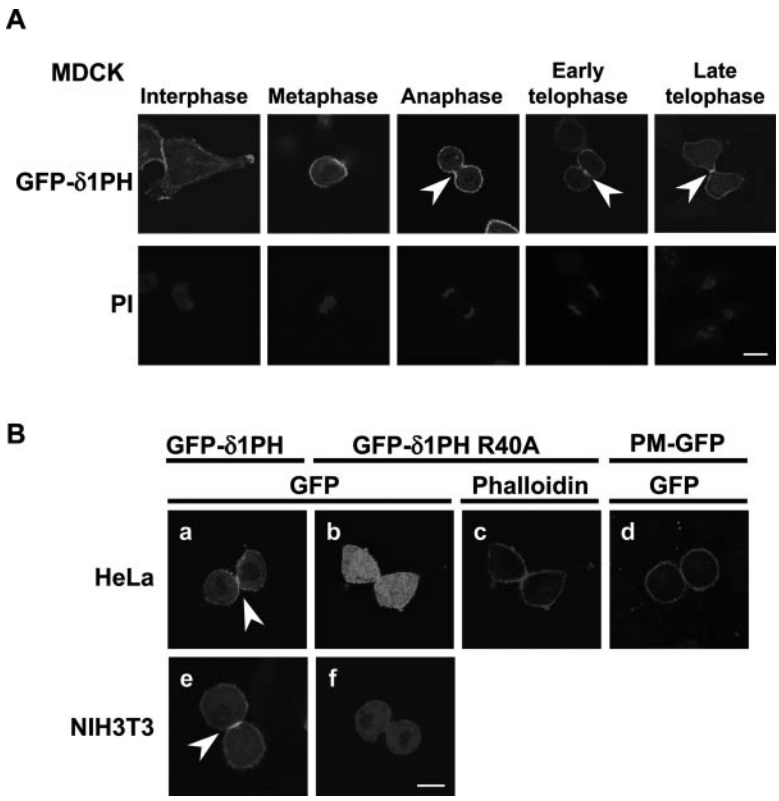

Fig. 1. **Localization of PIP_2 at the cleavage furrow detected by GFP-δ1PH.** (A) Localization of GFP-δ1PH during the cell cycle in MDCK cells. MDCK cells stably expressing GFP-δ1PH were synchronized with nocodazole and released by washing. At various stages of cell cycle, cells were fixed with 3% formaldehyde, stained with propidium iodide (PI) for nuclear visualization and analyzed by confocal microscopy. (B) Localization of GFP-δ1PH R40A compared with the wild type. HeLa cells expressing GFP-δ1PH (a) or GFP-δ1PH R40A (b, c: the same cells) were fixed and stained with Allexa-594 phalloidin to visualize F-actin (c). Localization of the plasma membrane–targeted GFP (PM-GFP) was examined as a control for the furrow targeting of GFP-δ1PH (d). Dividing NIH-3T3 cells transiently expressing GFP-δ1PH (e) or GFP-δ1PH R40A (f) were also shown. Arrowheads indicate apparent accumulation of GFP fluorescence at the cleavage furrow. Scale bar, 10 μm.

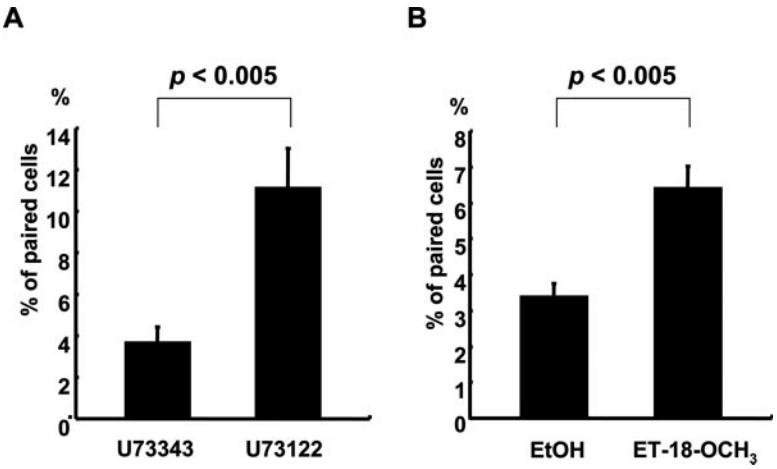

Fig. 2. **Effects of inhibitors of PIP_2 hydrolysis on cell division.** NIH-3T3 cells were incubated in a growth medium containing U73122 (A: 1 μM) or ET-18-OCH_3 (B: 30 μM), for 6 h. Two-paired cells in random fields were counted by phase-contrast microscopy. Significant differences (p values) were evaluated by counting 3 fields that contains at least 100 cells (Student's t-test).

δ1PH, in combination with δ1PH R40A, can therefore be used as a specific PIP_2 reporter (*18*). Initially, we tried to detect the PIP_2 localization during cytokinesis in MDCK cells as well as HeLa and NIH3T3 cells using GFP-fused δ1PH. GFP-δ1PH stably expressed in MDCK cells accumulates at the cleavage furrow and concentrated at the plasma membrane around the midbody in late telophase (Fig. 1A). Similar observation was obtained in HeLa cells stably expressing GFP-δ1PH. (Fig. 1B, a). Expression of GFP-δ1PH R40A resulted in uniform distribution of the fluorescence (Fig. 1B, b), indicating that the PH domain lacking the PIP_2 binding cannot target the furrow membrane even though cortical actin cytoskeleton at the furrow remains intact (Fig. 1B, c). The plasma membrane–targeted GFP probe, GFP with the myristoylation signal sequence of Lyn, stained only the peripheral edge of a dividing HeLa cell almost evenly but not the furrow (Fig. 1B, d). Similar observation was made in NIH-3T3 cells transiently expressing GFP-δ1PH and its mutant (Fig. 1B, e and f).

We also examined the necessity of PIP_2 hydrolysis for progression of cytokinesis. Treatment of NIH3T3 cells with U73122 (1 μM) significantly increased the number of paired cells, an indication of incomplete cell division, by 3-fold, compared with its inactive analogue U73343 (Fig. 2A). Similar results were obtained with another specific inhibitor for PIP_2 hydrolysis, ET-18-OCH_3 (30 μM); the number of paired cells was nearly doubled, compared with the control treatment (Fig. 2B). These results support previous reports demonstrating that PIP_2

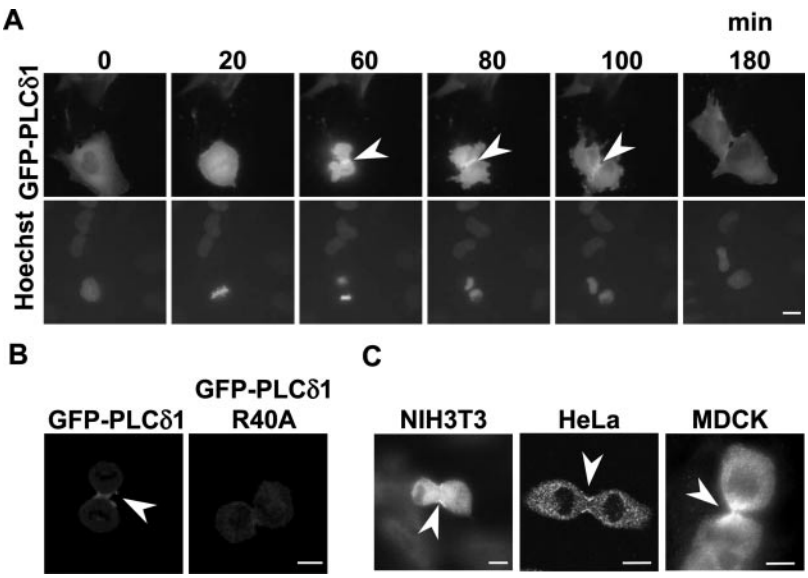

Fig. 3. **Localization of PLCδ1 during the cytokinesis.** (A) Time-lapse imaging of HeLa cells stably expressing GFP-PLCδ1. Cell nuclei were stained with Hoechst-33342. Arrowheads indicate apparent accumulation of GFP fluorescence at the furrow. Complete sequence of cytokinesis is shown in Supplementary Data Movie S1 (410 min in 42 frames). Similar time-lapse images of MDCK cells stably expressing GFP-PLCδ1 were also obtained (Supplementary Data Movie S2 and Movie S3 showing abscission of two daughter cells). (B) Images of HeLa cells expressing GFP-PLCδ1 (left) or GFP-PLCδ1 R40A (right) in telophase. (C) NIH3T3, HeLa and MDCK cells in telophase were fixed and endogenous PLCδ1 was detected by immunofluorescence using a polyclonal antibody raised against rat PLCδ1 (for NIH3T3) or a monoclonal antibody (for HeLa cells and MDCK cells) followed by FITC-labeled second antibodies. Arrowheads indicate apparent accumulation of fluorescence at the furrow. Scale bar, 10 μm.

is localized at the cleavage furrow of some fibroblastic and epithelial cell lines during cytokinesis (*10*, *22*) and that PIP_2 hydrolysis is necessary during fly cytokinesis (*15*, *16*).

In this study, we further investigated the localization of PLC isoforms using GFP-fused PLCs. First, distribution of PLCδ1 was traced during the cell cycle by time-lapse fluorescence microscopy using HeLa cells stably expressing GFP-PLCδ1 (Fig. 3A and Supplementary Data Movie S1). GFP-PLCδ1 started accumulating at the cleavage furrow from anaphase and the accumulation lasted towards late steps in cytokinesis especially to the dividing zone of two daughter cells. After abscission of two daughter cells, GFP-PLCδ1 was distributed mainly in lamellipodia. Nevertheless, apparent GFP fluorescence was not observed in the cytoplasmic bridge structure, which is thought to be composed of a bundle of microtubules surrounded by the plasma membrane and appears in the final step of cytokinesis. Similar results were obtained in MDCK cells stably expressing GFP-PLCδ1 (Supplementary Data Movies S2 and S3). In HeLa cells stably expressing GFP-PLCδ1 R40A, no apparent accumulation of the GFP fluorescence was observed at the furrow (Fig. 3B), indicating that it is the PH domain–PIP_2 interaction that plays a major role in the PLCδ1 targeting to the furrow. Endogenous PLCδ1 in NIH3T3, HeLa or MDCK cells also accumulates at the cleavage furrow in late telophase (Fig. 3C).

To explore the localization of other PLC isoforms during cytokinesis, several GFP-fused isoforms were expressed in HeLa cells. Among them, GFP-PLCβ1a and GFP-PLCβ1b, two splilcing variants of β1 isoform, and GFP-PLCδ3 were localized at the cleavage furrow during cytokinesis, whereas no accumulation of the GFP fluorescence was seen in cells expressing GFP-PLCβ2, GFP-PLCβ3 or GFP-PLCγ1 (Fig. 4A), although they remained intact during the experiment (Fig. 4B).

It has been reported that PLCδ3 interacts with PIP_2 *via* its PH domain (*23*). Therefore, PLCδ1 and PLCδ3 seem to share the same targeting mechanism to the furrow. On the other hand, the PH domain of PLCβ1 is less specific to PIP_2 and binds most strongly to phosphatidylinositol 3-phosphate [PI(3)P] (*24*). Targeting mechanisms of PLCβ1 to the furrow could therefore be different from those of PLCδ1 and PLCδ3. It is noteworthy that tubulin, which translocates to PIP_2-rich plasma membrane domains and interacts with both PLCβ1 and Gαq (*25*), accumulates in the furrow during cytokinesis.

Our results did not show clear localization of ectopically expressed PLCβ2 and PLCβ3 at the cleavage furrow. Normally, PLCβ2 binds to PLCδ1 and inhibits basal PLCδ1 activity (*26*). This interaction is suppressed by increasing Gβγ, suggesting that activated G protein–coupled receptor disrupts the PLCβ2-PLCδ1 complex. The liberated PLCδ1 may then be activated by Ca^{2+}, and plays a role as an amplifier of PLCβ signaling (*27*). Therefore, PLCβ2 should be excluded from regions where activation of PLCδ1 is needed.

The absence of PLCγ1 at the furrow is reasonable, since the PH domain of PLCγ1 binds to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 is generally localized at the leading edge of a migrating cell and dividing cells (*4*) and neither PIP_3 nor the kinase that produces PIP_3 from PIP_2 has been found in the furrow (*28*).

Our present study demonstrates for the first time that PLCδ1, PLCδ3 and PLCβ1 are localized at the cleavage furrow. These isoforms are thought to regulate levels of PIP_2 at the cleavage furrow for the normal progression of mitosis. Nevertheless, in the gene knockout mice for PLCδ1, PLCδ3 or PLCβ1, significant defects in cell growth have not been reported despite the fact that there are some alterations in phenotypes (*29–31*). Furthermore, overexpression of GFP-PLCδ1 E341A, a lipase-dead mutant, does not show a significant difference in the growth of HeLa or MDCK cells (not shown). These results suggest that PLC isoforms may compensate function of others or multiple isoforms are required for the progression of cytokinesis. Recently developed PLCδ1/PLCδ3 double knockout mice have been found to be embryonic lethal because placentae cannot be formed properly (*30*), suggesting that PLCδ1 and PLCδ3 share the same function essential for normal development of at least some tissues.

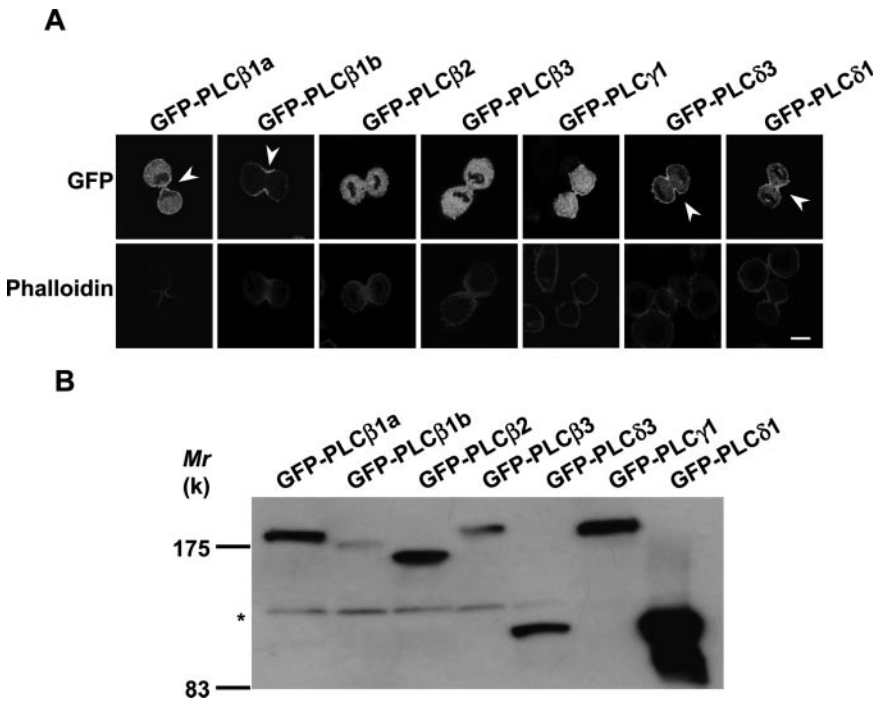

Fig. 4. **Localization of PLC isoforms during cytokinesis.** (A) GFP-fused PLCβ1a, PLCβ1b, PLCβ2, PLCβ3, PLCγ1 or PLCδ3 was transiently expressed in HeLa cells. HeLa cells stably expressing GFP-PLCδ1 are also used. Cells were synchronized with nocodazole. At 2 h after the cell-cycle release, cells were fixed and stained with Allexa-594 phalloidin to visualize F-actin and observed using confocal microscopy. Arrowheads indicate apparent accumulation of GFP fluorescence at the furrow. Scale bar, 10 μm. (B) The GFP-PLCs expressed in HeLa cells were checked by immunoblot analysis with an anti-GFP antibody. The asterisk denotes nonspecific bands.

Considering the close homology between the two isoforms, this seems reasonable.

Generally, animal cytokinesis has been described as a process based on constriction of an actomyosin-based contractile ring, acting at the cell periphery. When mammalian cells get into late M phase, a massive PIP_2 production would takes place as a result of the recruitment of active PI(4)P5K to the site of cell division. Ingression of the cleavage furrow should be under the strict influence of the actomyosin contractile ring, whose association to the plasma membrane is through PIP_2-enriched membrane domains. As the ring contracts, PIP_2-enriched domains should also be reduced, given the density of the membrane tether *via* PIP_2 unchanged. The PIP_2-hydrolysis may therefore be needed to reduce the size of the ring or release of the ring from the membrane surface before the end of cytokinesis. The PLC isoforms found at the furrow in this study could play a role in this PIP_2 reduction, leading to the disruption of the contractile ring.

Formation and disappearance of a unique lipid domain at the site of cell division seem crucial during cytokinesis. Emoto *et al.* have recently shown using CHO cells that PE is exposed on the cell surface of the cleavage furrow during late cytokinesis and that this PE movement is involved in regulation of the contractile ring disassembly by inactivation of RhoA, a regulator of the actin cytoskeleton, and PIP5Kβ, a RhoA effector (*22*).

Formation and disappearance of a unique lipid domain may also be important for membrane fusion events for completion of cell division. Gromley *et al.* demonstrated that secretary vesicle–mediated abscission occurs *via* formation of microtubule-directed recruitment of cytokinetic vesicles in the terminal phase of animal cytokinesis (*32*), although phosphoinositide does not seem directly involved in this process. In plant cell cytokinesis, however, phosphoinositide plays a certain role in vesicle fusion that is important for formation of the cell plate (*33*). Down regulation of Arabidopsis phosphatidylinositol 3-kinase resulted in severe growth defects (*34*), suggesting a crucial role for PI(3)P and its synthesis. It has recently been shown that late endosomal transport and vesicle fusion flagged by PI(3)P help the cell plate growth in Arabidopsis plants (*35*) and in tobacco BY-2 cells (*36*). Spatial regulation of PI(3)P synthesis and breakdown may be a key event in regulating cell plate growth during plant cytokinesis, but it is not clear whether PIP_2 is involved or not.

Finally, PIP_2 hydrolysis by active PLC isoforms at the cleavage furrow could also serve as a critical event in mobilizing intracellular Ca^{2+} by producing the second messenger IP_3. Ca^{2+} is essential for cytokinesis, since it also regulates remodeling of the actin cytoskeleton and possible vesicle fusion for membrane separation. For future study, it is of interest to examine whether cell lineage–specific isoforms of PLC, such as PLCβ4 and PLCγ2, and newly cloned isoforms, such as PLCε, PLCζ and PLCη, also play roles in cytokinesis.

Online Supplement available. Time-lapse imaging of dividing GFP-PLCδ1–expressing cells: [mvj209 Movie S1.mov] HeLa cells: 42 frames (10 min interval, total 410 min); [mvj209 Movie S2.mov] MDCK cells: 38 frames (3 min interval, total 111 min); [mvj209 Movie S3.mov] MDCK cell in telophase showing abscission of two daughter cells: 32 frames (2 min interval, total 62 min).

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