Supplemental Data

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

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Supplemental Experimental Procedures

Cell Culture and Transfection

NIH3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum, penicillin, and streptomycin. RAW 264.7 macrophages, 293T cells, and HeLa cells were grown in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. CHO cells were grown in RPMI supplemented with 10% fetal calf serum, penicillin, and streptomycin. NIH3T3, 293T, HeLa, and CHO cells were transfected with Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Transfections with "low" amounts of DNA used 0.20 to 0.50 μg of DNA per 35 mm dish. Transfections with "high" amounts of DNA used 2.0 to 5.0 μg of DNA per 35 mm dish. RAW macrophages were transfected with Fugene (Roche Applied Science, Indianapolis, IN) according to the manufacturer's directions.

Green Fluorescent Protein Fusion Constructs

pEGFP-N1, pEYFP-N1, and pEGFP-F vectors were obtained from Clontech (Palo Alto, CA). PLCδ-PH-EGFP and BTK-PH-EGFP were a generous gift of Tamas Balla (NIH). GFP-TubbyC and GFP-TubbyC-KKK were a generous gift of Lawrence Shapiro (Columbia University, New York, NY). The FAPP1-PH-EYFP and TAPP1-PH-EYFP constructs were purchased from Dario Alessi (University of Dundee, UK). We have described the p40PX-EYFP construct previously [S1]. The GFP-synaptojanin construct was a generous gift of Pietro de Camilli (Yale University, New Haven, CT). The EYFP fusion to the D227A mutant of PI(4)P-5-kinase was produced by amplification of the plasmid pEBB-HA-PIP5Kα-D227A [S2] and ligation into pEYFP-N1 digested with BglII and SalI. The effectiveness of the synaptojanin and PI(4)P-5-kinase D227A constructs at reducing PtdIns(4,5)P2 levels was assayed by measuring their effect on plasma membrane localization of PLCδ-PH reporters. In these control experiments (data not shown), synaptojanin reduced plasma-membrane-associated PLCδ-PH fluorescence by 45% ± 1% (n = 5), and PI(4)P-5-kinase D227A reduced levels by 46% ± 1% (n = 4).

Fluorescence Microscopy

Live-cell imaging was performed by epifluorescence with a Nikon Diaphot 300 with computer-controlled Uniblitz shutter (Vincent Associates, Rochester, NY) and a Photometrics Sensys CCD (Photometrics, Tucson, AZ) camera controlled by Image Pro Plus 4 software (Media Cybernetics, Silver Spring, MD). An environmentally controlled stage with regulated temperature (37°C) and atmosphere (humidified 10% CO_2) was used. Non-contact 20× and 40× objective lenses were used to minimize temperature fluctuations. Appropriate fluorescence filter sets were used for EGFP, EYFP, Texas Red, and DAPI (Chroma Technology, Rockingham, VT). Digital deconvolution processing was performed with Microtome 5.1 (Vaytek, Fairfield, IA). Time-lapse image series were assembled into movies with Quicktime software (Apple Computer, Cupertino, CA). Imaging was also performed on a Zeiss Axiovert inverted microscope (Zeiss) with an Axiocam, Axiovision 4.2 software, and appropriate fluorescent filter sets (Chroma Technology). Additional imaging was performed with a Perkin Elmer Ultraview spinning Nipkow disk confocal head with 488 nm and 568 nm laser lines mounted on a Nikon T2000U inverted microscope, and images were captured with a 100× oil immersion objective lens with an Orca ER cooled CCD camera with 2 × 2 binning controlled by Metamorph software.

Most of the experiments shown used unsynchronized cell populations imaged over long time periods (8–72 hr). Photoxicity was minimized by the use of short exposure times that were offset by the use of increased gain. Long exposures with low gain settings produced images with decreased pixilation but resulted in significant phototoxicity over the duration of the experiment. The use of short exposures with high gain allowed imaging of each of the EGFP fusion constructs for at least 72 hr without any evidence of phototoxicity.

Comparisons between different EGFP fusion constructs were performed with cells expressing similar levels of the fusion constructs. Expression levels were assessed by quantification of fluorescence either by flow cytometry for bulk populations or by quantification of fluorescence of images of single cells (for single-cell assays).

Cell Fixing and Staining

Cells grown on 25 mm round coverslips were washed once in PBS and then fixed in 3.7% paraformaldehyde in PBS (pH 7.4). For staining with Texas Red-phalloidin, the cells were permeabilized with 0.1% Triton X-100, blocked with 1% BSA/PBS/0.02% NaN3, and then stained for 20 min with Texas Red-phalloidin (4 U/ml, Molecular Probes, Eugene, OR) and DAPI (0.1 μg/ml, Sigma-Aldrich) in 1% BSA/PBS/0.02% NaN_3. Coverslips were then washed in PBS and mounted on slides with Fluoromount G (Southern Biotechnology, Birmingham, AL).

Hypotonic Treatment

CHO cells were synchronized at mitosis by treatment with 100 μM monastrol for 18 hr and then released by washout with complete cell-culture medium. Approximately 45 min later, the cells would synchronously begin to undergo cytokinesis as observed by time-lapse video microscopy. The media were abruptly replaced with water, and time-lapse images were obtained every 20 s. The rapid initial change in cleavage-furrow diameter was followed by measurement of the furrow diameter over the first 100 s. Relative cleavage-furrow diameter was calculated by division of the furrow diameter by the diameter at time zero for the same cell. The slope of the best-fit line representing the rate of change of the furrow diameter is graphed in Figure 2D. Drug treatments used 5 μM latrunculin B (Calbiochem) pre-treatment for 4 min or 5 μm jasplakinolide (Calbiochem) pre-treatment for 2 min.

Fluorescence Quantification

All quantitative comparisons were made on raw TIFF format images taken with identical exposure parameters. For comparison within a cell (poles versus cleavage furrow), cytosolic fluorescence was taken as background, and the net plasma membrane peak area was compared between measurements at the poles and at the cleavage furrow with ImageJ software (Wayne Rasband, NIH).

Statistics

All statistical comparisons were evaluated on a Macintosh computer with Statview 4 software (Abacus Concepts, Berkeley, CA). All values reported represent means ± standard error. Error bars in graphs also represent the standard error.

Supplemental References

S1. Kanai, F., Liu, H., Field, S.J., Akbary, H., Matsuo, T., Brown, G.E., Cantley, L.C., and Yaffe, M.B. (2001). The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. Nat. Cell Biol. *3*, 675–678.

S2. Tolias, K.F., Hartwig, J.H., Ishihara, H., Shibasaki, Y., Cantley, L.C., and Carpenter, C.L. (2000). Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. Curr. Biol. *10*, 153–156.