Molecular Biology of the Cell
Vol. 19, 3180–3191, August 2008

The PCH Family Member Proline-Serine-Threonine Phosphatase–interacting Protein 1 Targets to the Leukocyte Uropod and Regulates Directed Cell Migration

Kate M. Cooper,* David A. Bennin,† and Anna Huttenlocher†‡

*Cellular and Molecular Biology Program, Departments of ‡Medical Microbiology and Immunology and †Pediatrics, University of Wisconsin, Madison, WI 53706

Submitted February 29, 2008; Revised May 1, 2008; Accepted May 7, 2008
Monitoring Editor: Carole Parent

Pombe Cdc15 homology (PCH) family members have emerged as important regulators of membrane–cytoskeletal interactions. Here we show that PSTPIP1, a PCH family member expressed in hematopoietic cells, regulates the motility of neutrophil-like cells and is a novel component of the leukocyte uropod where it colocalizes with other uropod components, such as type I PIPKIγ. Furthermore, we show that PSTPIP1 association with the regulator of endocytosis, dynamin 2, and PSTPIP1 expression impairs transferrin uptake and endocytosis. We also show that PSTPIP1 localizes at the rear of neutrophils with a subpopulation of F-actin that is specifically detected by the binding of an F-actin probe that detects a more stable population of actin. Finally, we show that actin polymerization, but not the microtubule network, is necessary for the polarized distribution of PSTPIP1 toward the rear of the cell. Together, our findings demonstrate that PSTPIP1 is a novel component of the leukocyte uropod that regulates endocytosis and cell migration.

INTRODUCTION

Neutrophils are critical participants in the innate immune response to inflammatory stimuli such as tissue injury and infection. The inappropriate recruitment and retention of neutrophils within tissues is the hallmark of some chronic inflammatory diseases, such as autoinflammatory diseases, which are characterized by leukocyte infiltration into tissues including the joints and skin (Hull *et al.,* 2003). The recruitment of neutrophils to inflammatory sites requires a specialized form of directed cell migration in which neutrophils respond to gradients of chemoattractant with polarized actin polymerization and protrusion at the front of the cell (Devreotes and Janetopoulos, 2003; Niggli, 2003a; Parent, 2004). Substantial progress has been made in defining the components of the gradient sensing machinery at the leading edge of the cell that mediate the formation of a dominant pseudopod with localized and dynamic actin polymerization at the leading edge. However, less is known about what regulates the uropod of the cell where dynamic actin polymerization is inhibited and localized RhoA-mediated actomyosin contractility mediates rear release (Niggli, 1999; Eddy *et al.,* 2000).

Cell polarization is necessary for neutrophil motility and can occur in either a gradient or uniform concentration of chemoattractant. In both cases, highly dynamic actin rapidly assembles and disassembles at the leading edge, whereas cortical actin is oriented toward the rear of the neutrophil. Accordingly, some types of actin-binding proteins such as Wiskott-Aldrich syndrome protein (WASP) have been reported to localize to both the front and rear of the cell during polarized cell migration (Myers *et al.,* 2005), whereas other actin-binding proteins, such as Arp3, specifically localize at the areas of dynamic actin-based protrusions at the cell front (Weiner *et al.,* 2007). Actin cytoskeleton dynamics are tightly regulated by actin regulatory proteins including the polarized activity of members of the Rho GTPase family, with Rac and Cdc42 active at the leading edge of the cell and Rho toward the rear of the cell (reviewed in Niggli, 2003b). The asymmetric recruitment and activation of actin regulatory proteins is critical for directed cell migration, and a key event in mediating this polarized distribution is the contribution of phosphoinositide signaling. For example, previous work has shown that phosphoinositide 3-kinase and its product phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) accumulate at the leading of edge of the cell (Parent *et al.,* 1998; Servant *et al.,* 2000). Recent work from our lab and other labs localizes type I phosphatidylinositol phosphate kinases (PIPKI) to the uropod of the cell where these enzymes likely contribute to the asymmetric accumulation of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) at the rear of the cell (Lacalle *et al.,* 2007; Lokuta *et al.,* 2007).

There is continued interest in understanding the signaling machinery that contributes to asymmetric dynamics of membrane–cytoskeletal interactions during directed cell migration. An attractive candidate for mediating these interactions during polarized cell migration is the Pombe Cdc15 homology (PCH) family of adaptor proteins that has been

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–02–0225) on May 14, 2008.

Address correspondence to: Anna Huttenlocher (huttenlocher@wisc.edu).

Abbreviations used: C5a, complement factor 5a; DIC, differential interference contrast microscopy; dHL-60, differentiated HL-60; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PAPA, pyogenic sterile arthritis pyoderma gangrenosum and acne; PCH, Pombe Cdc15 homology; PIPKIγ, typeIγ phosphatidylinositol phosphate kinase; PSTPIP1, proline-serine-threonine phosphatase–interacting protein 1; SH3, Src homology 3; WASP, Wiskott-Aldrich syndrome protein; Utr-CH, calponin homology domain of utrophin.

implicated in regulating membrane–cytoskeletal interactions and actin dynamics. PCH family proteins contain an amino (N)-terminal F-BAR (FCH [FER/CIP4 homology] and BAR [Bin/amphiphysin/Rvs]) domain, also called an EFC (extended FCH domain) that binds to phospholipids, most strongly to PtdIns(4,5)P_2, and induces membrane tubulation (Tsujita *et al.*, 2006). Many PCH family proteins also contain a carboxyl (C)-terminal Src homology 3 (SH3) domain that mediates protein–protein interactions. The SH3 domains of many of the PCH family members bind to WASP and dynamin (Cote *et al.*, 2002; Tsujita *et al.*, 2006). Accordingly, PCH family proteins have been implicated both in regulating F-actin assembly and bundling (Chitu *et al.*, 2005) and in endocytosis (Itoh *et al.*, 2005; Tsujita *et al.*, 2006).

The PCH family member proline-serine-threonine phosphatase–interacting protein 1 (PSTPIP1) was first described as an adaptor protein that interacts with the PEST-sequence Protein Tyrosine Phosphatase (PTP-PEST; Spencer *et al.*, 1997) and is required for the dephosphorylation of WASP by PTP-PEST (Cote *et al.*, 2002), thereby likely having an inhibitory effect on actin polymerization (Torres and Rosen, 2003). A single amino acid substitution in the gene encoding PSTPIP1 leads to the human autoinflammatory disease PAPA (pyogenic sterile arthritis, pyoderma gangrenosum, and acne) syndrome (Wise *et al.*, 2002). The disease is characterized by recurrent, destructive inflammation of the skin and joints. Patient tissues develop sterile, pyogenic, neutrophil-rich material, indicating a possible link between PSTPIP1 and normal neutrophil function. Unlike other PCH family members, which are ubiquitously expressed, PSTPIP1 is expressed primarily in hematopoietic cells (Chitu and Stanley, 2007). PSTPIP1 interacts with other adaptor proteins, such as pyrin (Shoham *et al.*, 2003), that are components of an inflammasome complex that mediates the generation of activated Interleukin (IL)-1β (recently reviewed in Church *et al.*, 2008). Two other autoinflammatory diseases, familial Mediterranean fever (FMF; Babior and Matzner, 1997) and neonatal onset multisystem inflammatory disease (NOMID; Neven *et al.*, 2004), are also caused by mutations in proteins, pyrin and NLRP3 (also called NALP3 or cryopyrin), respectively, that are associated with the inflammasome. Interestingly, previous studies have shown that neutrophils isolated from patients with mutations in NLRP3 display defects in neutrophil chemotaxis (Huttenlocher *et al.*, 1995; Lokuta *et al.*, 2005), suggesting that members of the inflammasome complex may play roles in neutrophil motility. Here, we sought to investigate the role of PSTPIP1 in neutrophil motility and to investigate its temporal and spatial regulation during directed leukocyte migration.

MATERIALS AND METHODS

Expression Constructs

The following constructs were kind gifts: myc-tagged human PSTPIP1 in pcDNA3.1 from Daniel Kastner (National Institutes of Health, NIAMS, Bethesda, MD), rat dynamin 2 aa-green fluorescent protein (GFP) and its K44A mutant (in pEGFP-N1 from Clontech, Palo Alto, CA) from Mark McNiven (Mayo Clinic, Rochester, MN; Cao *et al.*, 1998), Arp3-GFP from Orion Weiner (University of California, San Francisco; Weiner *et al.*, 2007), mCherry-actin from Gerald Marriott (University of Wisconsin, Madison), and RFP-Utr-CH from William Bement (University of Wisconsin, Madison; Burkel *et al.*, 2007). PSTPIP1 was subcloned into the pEGFP-C1 vector (Clontech) to generate GFP-PSTPIP1. GFP-PSTPIP1 was then further cloned into the retroviral vector pMX IRES-GFP which had the IRES-GFP removed, for use in expressing the GFP-PSTPIP1 fusion in HL-60 cells. The construct GFP-PSTPIP1-ΔSH3 involved a deletion of amino acids 360 through 416. PSTPIP1 was also subcloned into pmCherry-C, which was made as described (Lokuta *et al.*, 2007) from mCherry that was a kind gift from Roger Tsien (University of California, San Diego). pEGFP-Actin was purchased from Clontech. pmCherry-PIPKIγ661 was previously described (Lokuta *et al.*, 2007). Human N-WASP was cloned into pcDNA 3.1 flag using the following primers: Fwd: 5-CTAGTAGAATTCATGAGCTCCGTCCAGCAG-3 and Rev: 5-CAAGGTCTCGAGGTCTTCCCACTCATCATC-3.

Antibodies and Reagents

Fibronectin was purified from human plasma by affinity chromatography as described (Ruoslahti *et al.*, 1982). DMEM/F12 was obtained from Invitrogen (Carlsbad, CA); fibrinogen, fatty-acid-free bovine serum albumin (BSA), latrunculin B, and Complement factor 5a (C5a) were from Sigma-Aldrich (St. Louis, MO); anti-mouse IgG (ChromaPure; whole molecule) and FITC goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA); rhodamine-transferrin, anti-GFP, jasplakinolide, and AlexaFluor 680 goat-anti-mouse IgG secondary antibody were purchased from Molecular Probes (Invitrogen-Molecular Probes, Carlsbad, CA); and IRDye 800CW goat anti-rabbit IgG secondary antibody was obtained from Rockland Immunochemicals (Gilbertsville, PA). Blebbistatin was purchased from Calbiochem (203390, EMD Biosciences, San Diego, CA). The anti-p38 antibody was from Invitrogen (Carlsbad, CA). The anti-PSTPIP1 antibody was generated in rabbits by our laboratory as previously described (Bennin *et al.*, 2002), using the full-length PSTPIP1 protein fused to glutathione *S*-transferase (GST) bound to glutathione beads (Clontech). Antibodies cross-reactive to GST were removed from the anti-sera by negative selection and then antibodies for PSTPIP1 were affinity-purified over a covalently linked GST-PSTPIP1 column.

Primary Neutrophil Isolation

C57Bl/6 mice (Harlan Sprague-Dawley, Madison, WI) were killed in accordance with an institution-approved protocol, and the bone marrow–derived neutrophils were isolated, nucleofected, and maintained as previously described (Lokuta *et al.*, 2007).

Primary human neutrophils used for endogenous protein localization analysis were obtained from healthy donors as described previously (Lokuta *et al.*, 2003).

Cell Culture, Transfection, and Infection

HL-60 cells (UCSF tissue culture facility) were cultured and differentiated as previously described (Nuzzi *et al.*, 2007). Phoenix viral packaging cells were transiently transfected by calcium-phosphate precipitation, and viral supernatant was harvested and used to retrovirally infect HL-60 cells as previously described (Nuzzi *et al.*, 2007). Populations of GFP-positive cells were obtained by fluorescence-activated cell sorting and verified for expression by immunoblotting.

HL-60 cells were transiently transfected by nucleofection using the following protocol: On day 6 of differentiation, cells were counted, and 2×10^6 cells per nucleofection were spun down at $75 \times g$ and resuspended in 100 μl of nucleofection solution V (Amaxa Biosystems, Gaithersburg, MD). Cell solution was immediately transferred to a nucleofection cuvette with 1 μg of DNA (1 μg of each DNA was used for double transfections). Cells were nucleofected using a Nucleofector II (Amaxa) program T- 19. After nucleofection, the cells were transferred to a 12-well plate containing 1 ml HL-60 media with heat-inactivated serum (Iscove's modified Dulbecco's medium [ATCC, Manassas, VA], 10% heat-inactivated fetal bovine serum [FBS], 100 U/ml penicillin, and 100 g/ml streptomycin; Cellgro, Herndon, VA). Cells were incubated at 37°C in 5% CO_2 for 2 h before using for imaging of random migration in Gey's media (20 mM HEPES, 132 mM NaCl, 1 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, and 0.5% human serum albumin; American Red Cross Blood Services, Washington, DC) after treating with 10 mM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma-Aldrich), as detailed below.

HeLa and HEK 293 cells were maintained in DMEM supplemented with 10% FBS, 4.5 μg/L glucose, 2 mM L-glutamine, 1% nonessential amino acids (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection of HEK 293 cells was performed by calcium-phosphate precipitation as previously described for Pheonix cells (Nuzzi *et al.*, 2007). Transfection of HeLa cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Experiments were performed 24–48 h after transfection.

Transwell Migration Assay

Transwell filters (6.5 mm, 3-μm pores, Costar #3415; Corning, Corning, NY) were coated on the top and bottom with 2.5 μg/ml fibrinogen in DPBS for 1 h at 37°C. Filters were dried after coating. Differentiated HL-60 (dHL-60) cells (4×10^5) were plated in the top chamber in Gey's media. Gey's media with 11.25 nM C5a, 10 nM fMLP, or Gey's media alone was added to the bottom chamber. Cells were allowed to migrate for 3 h at 37°C and 5% CO_2. EDTA was then added to the bottom chamber to yield a final concentration of 45 mM. The plate was placed at 4°C for 15 min, and then the top chamber was removed. The cells that migrated to the bottom well were counted by flow cytometry and expressed relative to control. Statistical significance was calculated in GraphPad Prism (San Diego, CA) using analysis of variance with a Bonferroni posttest.

Live Fluorescence Microscopy and Chemotaxis

Fluorescence imaging of live cells was performed using a Nikon 60× differential interference contrast (DIC) oil immersion objective (NA = 1.40) on a Nikon Eclipse TE300 inverted fluorescent microscope (Melville, NY). Images were acquired every 15 s for 15 min with a Hamamatsu cooled CCD video camera (Hamamatsu Photonics, Bridgewater, NJ) housed in a closed system to maintain the temperature at 37°C. Glass-bottom dishes were acid-washed and then coated with a combination of 2.5 μg/ml fibrinogen and 10 μg/ml fibronectin. Cells were treated with 10 mM fMLP for random imaging. For gradient generation in the chemotaxis dHL-60 cell movies, C5a was loaded into an Eppendorf femptotip, and a gradient was formed by slow release of the chemoattractant from the tip into the medium using an Eppendorf FemptoJet microinjection system (Westbury, NY) with a constant back pressure of 10 hPa as described previously (Servant *et al.*, 1999; Lokuta *et al.*, 2003). MetaMorph Imaging software (Universal Imaging, Downingtown, PA) was used for acquisition and analysis.

Immunocytochemistry and Endocytosis

For HeLa cells, glass coverslips were acid-washed and coated with 5 μg/ml fibronectin. HeLa cells were plated on the coverslips in complete medium and allowed to adhere for 4–6 h. For immunocytochemistry cells were fixed, permeabilized, and stained as described previously (Huttenlocher *et al.*, 1996). For human primary neutrophil immunocytochemistry, cells were plated on acid-washed coverslips coated with 10 μg/ml fibrinogen in DPBS, treated with 100 nM fMLP, and allowed to adhere for 10 min. Cells were fixed with 3.7% formaldehyde in PBS for 15 min, quenched with 0.15 M glycine for 15 min, and then treated with 0.2% Triton X-100 (Fisher Scientific, Pittsburgh, PA) to permeabilize for 15 min. Nonspecific binding was blocked with PBS containing 10% heat-inactivated FBS at 4°C overnight. Cells were stained for 30 min with anti-PSTPIP1 antibody and then FITC goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody. All antibody incubations were performed at room temperature, and cells were washed extensively in PBS between each incubation step.

The fluorescent transferrin uptake assay was performed as described (Itoh *et al.*, 2005), with the following modifications: after the cells were plated on the coated coverslips for 4 h, the normal medium was exchanged for DMEM plus 0.2% fatty-acid free BSA. The cells were then serum-starved for 45 min and incubated with 30 μg/ml rhodamine-conjugated transferrin (Molecular Probes) for 10 min. After acid washing with a solution of 150 mM NaCl and 10 mM HCl, pH = 2.0 for 2 min (Engqvist-Goldstein *et al.*, 2004), cells were fixed (but neither permeabilized or stained) as previously described (Huttenlocher *et al.*, 1996).

Fluorescence signal of cells were visualized using a Nikon 60× DIC oil immersion objective (NA = 1.40) on a Nikon Eclipse TE300 inverted fluorescent microscope. Fluorescence images were digitally acquired using a cooled charge-coupled device video camera (Hamamatsu Photonics) and processed with Metamorph (Universal Imaging). Images are representative of a minimum of 25 cells from at least three independent experiments performed in duplicate. The transferrin uptake of the transfected cells was compared with untransfected cells on the coverslip and scored as being similar to the average fluorescence of untransfected cells ("positive" for performing transferrin endocytosis) or having no fluorescence above background (not performing endocytosis) and expressed relative to control GFP cells (set as 100%). GFP-control cells had between 85 and 96% transferrin uptake in the experiments. More than 25 cells were counted for each of three independent experiments. Statistical significance was calculated in GraphPad Prism using analysis of variance with a Tukey posttest.

Coimmunoprecipitation

HEK 293 cells were used 24 h after transfection, washed once with PBS, and scraped into lysis buffer (1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-base at pH 8.0, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 200 nM phenylmethanesulphonyl fluoride, and 100 μM sodium vanadate) on ice and clarified by centrifugation. Total lysate (500–800 g) was added to 30 μl of 50% slurry of protein A-Sepharose (RepliGen, Needham, MA) and 3 μg of anti-mouse IgG, or 3 μg of anti-myc antibody (Cell Signaling Technology, Danvers, MA) . After 2-h incubation at 4°C with tilting, samples were washed three times with 1% NP-40 buffer and then extracted with SDS sample buffer. Extracts were resolved on 4–20% gradient SDS-PAGE gels and transferred to nitrocellulose for Western blot analysis. Anti-PSTPIP1 (1:100) or anti-GFP (Invitrogen-Molecular Probes; 1:500) were used for protein detection.

Western Blotting

For Western blots, cells were lysed in 1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-base at pH 8.0, with protease-inhibitor cocktail (P-8340, Sigma-Aldrich), phosphatase-inhibitor cocktail (P-5725, Sigma-Aldrich), 2 mM phenylmethylsulfonyl fluoride, 100 μM sodium orthovanadate, 900 μM benzamidine, and 1 mM phenanthroline on ice and clarified by centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Equal amounts of total protein were denatured in SDS sample buffer, resolved on 4–20% gradient SDS-PAGE gels and transferred to nitrocellulose using standard methods. Primary antibodies were used at 1:100 and 1:1000 for anti-PSTPIP1 and anti-p38, respectively. Anti-GFP was used at 1:500. Secondary antibodies were used at 1:20,000. Western blots were imaged with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Far Western

^{35}S (MP Biomedicals, Irvine, CA) labeled PSTPIP was generated from the pcDNA 3.1-PSTPIP1-myc construct as described in the Promega TnT T7 coupled reticulocyte lysate system (L4610; Madison, WI). Exogenously expressed GFP, GFP-tagged dynamin 2 or flag-tagged N-WASP proteins were isolated by immunoprecipitation from HEK 293 cells lysed with NP-40 lysis buffer (50 mM HEPES, 75 mM NaCl, 1% NP-40, and 10% glycerol) using 1 μl anti-GFP (Invitrogen-Molecular Probes) or 1 μl anti-Flag M2 (Sigma-Aldrich), respectively. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were denatured/renatured by serial washing 15 min each at 4°C with 6 M, 3 M, 1.5 M, 0.75 M, 0.375 M, 0.188 M, 0 mM guanidine HCL in a HEPES buffer (25 mM HEPES, 25 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM DTT). Membrane was then blocked with 3% nonfat dry milk in KCl buffer (142.5 mM KCL, 5 mM $MgCl_2$, 10 mM HEPES, and 0.2% NP-40) 1 h at room temperature and then incubated with probe diluted 1:4 in KCl buffer for 3 h at room temperature. Membrane was washed in KCL buffer extensively and dried. After an overnight incubation, bound probe was visualized using the STORM PhosphoImager system (GE Healthcare Life Sciences, Piscataway, NJ).

RESULTS

PSTPIP1 Localizes to the Uropod and Regulates Chemotaxis in dHL60 Cells

Previous studies have indicated that PSTPIP1 is expressed primarily in hematopoietic cells. To determine if HL-60 cells, a neutrophil-like cell line, express PSTPIP1, we generated a polyclonal antibody to human PSTPIP1. We found that PSTPIP1 was expressed at low levels in nondifferentiated HL-60 cells and was significantly up-regulated when HL-60 cells were differentiated with DMSO into neutrophil-like cells (dHL-60; Figure 1A). We also found that both primary neutrophils and macrophages express endogenous PSTPIP1 by Western analysis (data not shown). We next determined the localization of endogenous PSTPIP1 using immunofluorescence in human primary neutrophils treated with a uniform concentration of the chemoattractant fMLP and found that endogenous PSTPIP1 localized at the uropod of primary neutrophils (Figure 1B).

To further characterize the localization of PSTPIP1, we generated dHL-60 cells that stably express GFP-PSTPIP1. In a gradient of chemoattractant, GFP-PSTPIP1 localized dynamically at the uropod of the cell during chemotaxis. Similar results were obtained with primary mouse neutrophils transduced with GFP-PSTPIP1 (data not shown). To determine the temporal relationship between chemoattractant stimulation and PSTPIP1 localization to the uropod, time-lapse images were obtained after the initial application of chemoattractant. In general, PSTPIP1 localized to the uropod after the initial pseudopod was formed at the leading edge of the cell, and the cell developed a polarized morphology (Figure 1C), suggesting that PSTPIP1 targeting to the cell rear required the cell to be polarized. Accordingly, during the migratory response, when cells paused or lost polarity PSTPIP1 was no longer localized away from the highest concentration of chemoattractant and displayed a more uniform localization along the membrane in punctate dots (see Supplementary Material, Figure 1.mov). To further characterize the determinants of PSTPIP1 targeting, we expressed GFP-PSTPIP1 that lacked the SH3 domain. We found that GFP-PSTPIP1-ΔSH3 also localized to the uropod (Supplementary Materials, Figure S1), suggesting that binding to proteins such as WASP is not necessary for the targeting of PSTPIP1 to the rear of the cell.

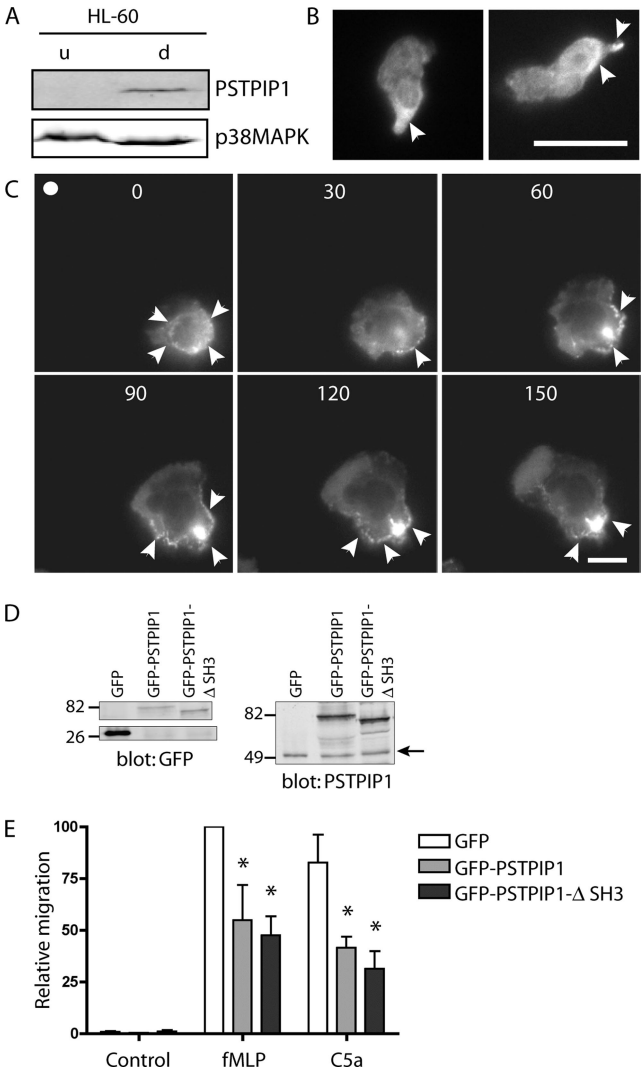

Figure 1. PSTPIP1 localizes to the uropod and regulates cell migration. (A) Immunoblot showing endogenous PSTPIP1 expression in either undifferentiated (u) or differentiated (d) HL-60 cells. Blotting for p38 MAPK shows equal loading. (B) Representative images of human primary neutrophils treated with 100 nM fMLP show localization of endogenous PSTPIP1 toward the rear of the cell. Arrowheads show PSTPIP1 localization at the uropod. Bar, 10 μm. (C) Representative fluorescence time-lapse images of dHL-60 cells that stably express GFP-PSTPIP1. Cells were plated as described in *Materials and Methods* and exposed to a chemotactic gradient generated by the slow release of C5a from a Femptotip micropipette. Fluorescent time-lapse images were taken at 30-s intervals after exposure to the micropipette. The direction of the micropipette is indicated by a solid white circle. Arrowheads indicate GFP-PSTPIP1 localization. Bar, 10 μm. Shown are representative images from at least three experiments. (D) Immunoblot showing expression of GFP, GFP-PSTPIP1, and GFP-PSTPIP1-ΔSH3 in HL-60 stable cell lines. Differentiated HL-60 cell lines that express GFP, GFP-PSTPIP1 or GFP-PSTPIP1-ΔSH3 were lysed as described in *Materials and Methods* and probed for GFP or PSTPIP1 as indicated. Arrow indicates endogenous protein expression. (E) Transwell assay was performed in the absence of stimuli (Control) or with C5a or fMLP in the bottom chamber for 3 h using dHL-60 cell lines that stably express control vector (GFP), GFP-PSTPIP1, or GFP-PSTPIP1-ΔSH3. Migration is shown relative to control cells migrating to fMLP (100%). Means ± SEM is shown from four independent experiments. Significant difference compared with control; *$p < 0.01$, two-way ANOVA, Bonferroni posttests.

To determine if PSTPIP1 regulates neutrophil chemotaxis, dHL-60 cells that stably express GFP alone, GFP-PSTPIP1, or GFP-PSTPIP1-ΔSH3 (Figure 1D) were assayed for cell migration by transwell assay. We observed a substantial impairment in the chemotaxis of dHL-60 cells that express GFP-PSTPIP1 or GFP-PSTPIP1-ΔSH3, compared with GFP alone, in response to both C5a and fMLP using transwell assay (Figure 1E). Despite substantial effort, we were not able to successfully knock down endogenous PSTPIP1 expression in dHL-60 cells. However, previous studies have suggested that targeted knockdown and overexpression of PCH family members have similar effects on basic cellular functions such as endocytosis (Tsujita *et al.*, 2006). Accordingly, expression of wild-type PSTPIP1 and a putative dominant negative form that lacks the SH3 domain, PSTPIP1-ΔSH3, had similar effects on chemotaxis by transwell assay. To identify a mechanism for the migration defect time-lapse microscopy was performed in a gradient of C5a. The cells that expressed wild-type PSTPIP1 displayed a reduced velocity of migration of 233 μm/h compared with the control average velocity of 302 μm/h ($p < 0.01$, paired Student's *t* test). However, there was no obvious morphological defect in the cells that expressed either wild-type PSTPIP1 or the PSTPIP1-ΔSH3 (data not shown). Together, these findings suggest that PSTPIP1 is a novel component of the neutrophil uropod that regulates the efficiency of neutrophil-directed cell migration induced by chemoattractant stimulation.

PSTPIP1 Colocalizes with TypeIγ PIPK at the Uropod during dHL-60 Motility

We next wanted to determine if PSTPIP1 colocalized with other uropod markers during chemotaxis. We recently showed that typeIγ PIPK (PIPKIγ) localizes to the uropod of neutrophils and dHL-60 cells during chemotaxis (Lokuta *et al.*, 2007). PIPKIγ has also been implicated in vesicular trafficking and in the regulation of endocytosis (Bairstow *et al.*, 2006). To address this question, dHL-60 cells that stably expressed GFP-PSTPIP1 were transiently transfected with mCherry-PIPKIγ using nucleofection. Live time-lapse imaging demonstrated that in a uniform concentration of chemoattractant dHL-60 cells polarize and migrate and that PSTPIP1 and PIPKIγ showed similar distributions and dynamics at the rear of the cell during random migration (Figure 2). Together, our findings indicate that PSTPIP1 is dynamically localized to the uropod of neutrophil-like cells, where it colocalizes with another uropod protein PIPKIγ.

PSTPIP1 Interacts Directly with Dynamin and Regulates Endocytosis

Recent studies suggest a critical role for PCH family members in the regulation of endocytosis through their interactions with dynamin (Itoh *et al.*, 2005). In addition, it has previously been shown that endocytosis of surface receptors, such as integrins, occurs at the rear of the cell during neutrophil motility (Pierini *et al.*, 2000); however, few studies have addressed the localization of endocytosis machinery during neutrophil chemotaxis. A recent study in fact suggests that disruption of endocytosis at the uropod impairs T-cell migration (Samaniego *et al.*, 2007). Because PSTPIP1 is at the rear of neutrophils, we were interested in determining if PSTPIP1 colocalizes and interacts with dynamin. Previous studies indicate that the SH3 domain alone of PCH family members, including PSTPIP1, interacts with dynamin 2 (Tsujita *et al.*, 2006). To determine if PSTPIP1 and dynamin 2 colocalize we overexpressed both mCherry-PSTPIP1 and GFP-dynamin2 in HeLa cells and dHL-60 cells. In HeLa cells

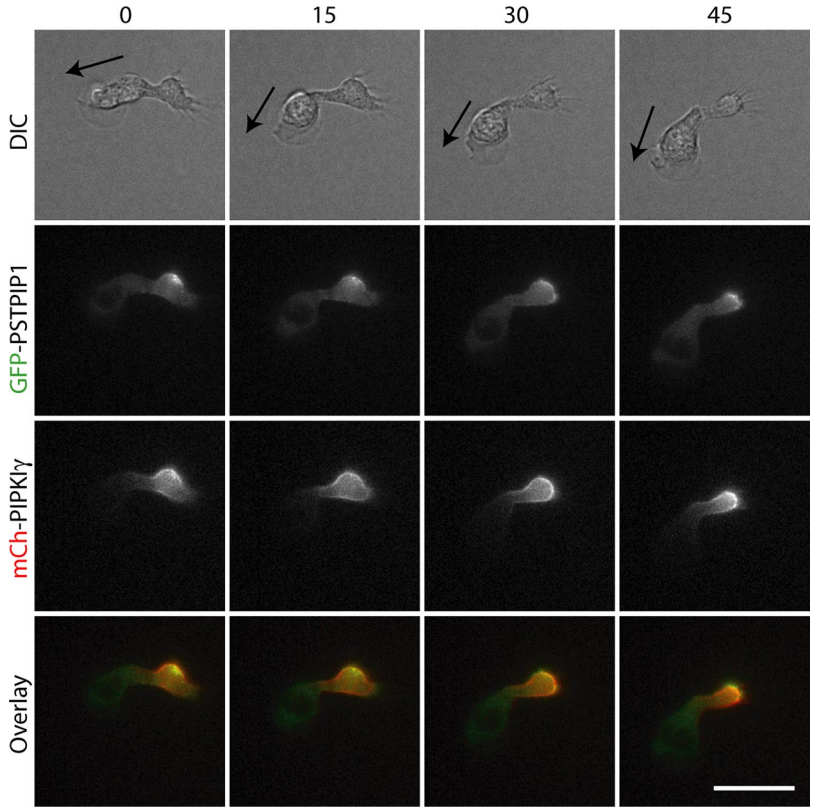

Figure 2. PSTPIP1 and PIPKIγ661 colocalize at the uropod in dHL-60 cells. Representative DIC and fluorescence images of dHL-60 cells that stably express GFP-PSTPIP1 and transiently express mCherry-PIPKIγ661 stimulated with a uniform concentration of 10 nM fMLP. Images were taken at 15-s intervals as indicated. Bar, 10 μm. Arrows show the direction of cell migration.

both dynamin 2 and PSTPIP1 colocalized at membrane tubules (Figure 3A, top and middle). Interestingly, both dynamin 2 and PSTPIP1 also colocalized at the uropod of dHL-60 cells (Figure 3A, bottom). Previous studies have reported that the SH3 domain of PSTPIP1 interacts with dynamin 2. To determine if full-length PSTPIP1 interacts with dynamin 2, coimmunoprecipitation analysis was performed. We found that dynamin 2 coimmunoprecipitated with full-length PSTPIP1, but not with GFP alone (Figure 3B) or mouse IgG control (data not shown). Furthermore, far Western analysis showed that the radiolabeled PSTPIP1 probe bound to the flag-N-WASP control and to GFP-dynamin 2, but not to GFP alone (Figure 3C). Taken together these data indicate that PSTPIP1 and dynamin 2 directly interact and colocalize at the uropod in dHL-60 cells.

Because PSTPIP1 binds directly to dynamin and colocalizes with dynamin 2 within cells, we next determined if PSTPIP1 regulates endocytosis. Although PCH family members have been implicated in endocytosis (Tsujita *et al.*, 2006), no previous studies have determined if PSTPIP1 regulates endocytosis. HeLa cells were transiently transfected with GFP, GFP-PSTPIP1, GFP-PSTPIP1-ΔSH3, GFP-dynamin 2, or GFP-dynamin 2-K44A, a dominant negative form that has previously been shown to inhibit endocytosis (Herskovits *et al.*, 1993). We found that expression of PSTPIP1 in HeLa cells inhibited endocytosis of transferrin to the same extent as expression of GFP-dynamin 2-K44A (Figure 4). However, cells that express GFP-PSTPIP1-ΔSH3 showed no defect in endocytosis, suggesting that SH3-binding proteins such as dynamin are critical for the effect of PSTPIP1 on endocytosis. Together, these data support a critical role for PSTPIP1 in regulating endocytosis through its SH3 domain.

PSTPIP1 Does Not Colocalize with Dynamic Actin at the Leading Edge of the Cell

Substantial evidence suggests that PCH family proteins regulate the actin cytoskeleton and interact with actin-binding proteins such as WASP (Itoh *et al.*, 2005; Tsujita *et al.*, 2006). Furthermore, the related protein PSTPIP2, which lacks a SH3 domain and is not expressed in neutrophils (Grosse *et al.*, 2006), has been reported to target to areas of membrane protrusion in macrophages such as filopodia and to enhance their formation (Chitu *et al.*, 2005). Previous studies also report that PSTPIP1 binds to WASP and regulates its activity (Cote *et al.*, 2002; Badour *et al.*, 2003). To determine if PSTPIP1 colocalizes with dynamic actin in the cell during migration, we transiently expressed mCherry-actin in dHL-60 cells that stably express GFP-PSTPIP1 or GFP alone. The mCherry-actin showed dynamic localization mainly at the leading edge of the cell during migration in a uniform concentration of chemoattractant (Figure 5). Interestingly, mCherry-actin did not colocalize with PSTPIP1, which was found at the uropod during active migration. Together, these findings suggest that PSTPIP1 does not colocalize with dynamic actin at the leading edge of the cell. This is in contrast to the closely related actin-binding protein PSTPIP2 that targets to areas of membrane protrusion in macrophages (Chitu *et al.*, 2005).

Actin-binding Domain of Utrophin Targets to the Uropod during dHL-60 Motility and Colocalizes with PSTPIP1

A recent report by Burkel *et al.*, (2007) characterized the development of a novel probe to detect the dynamics of F-actin in cells (Burkel *et al.*, 2007). The probe contains the calponin homology domain of utrophin fused to red fluorescent protein (RFP; RFP-Utr-CH), which binds to F-actin

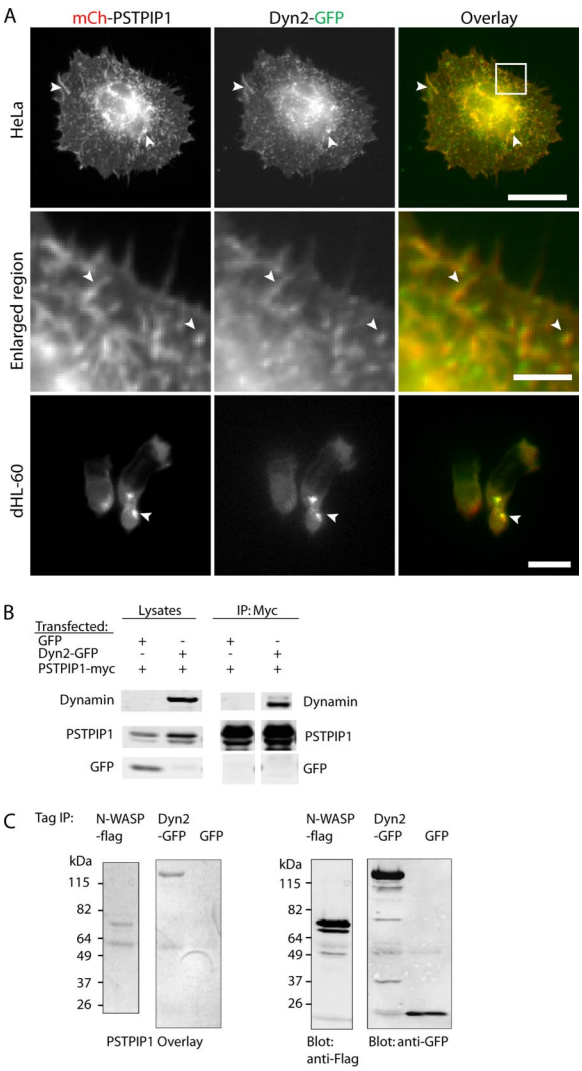

Figure 3. PSTPIP1 colocalizes and interacts with dynamin 2. (A) Representative images of HeLa cells (top panels) and dHL-60 cells (dHL-60; bottom panels) that transiently express mCherry-PSTPIP1 and GFP-dynamin 2. The HeLa cells were fixed as described in *Materials and Methods* and fluorescence images are shown of the PSTPIP1 (red) and Dynamin 2 (green) and an overlay to show colocalization. Bar, 30 μm. A magnified region of the cell is shown (enlarged region). Bar, 10 μm. Time-lapse images of the dHL-60 cells were taken as described above in the presence of a uniform concentration of fMLP with PSTPIP1 (red), dynamin2 (green), and an overlay. Bar, 10 μm. Arrowheads indicate areas of colocalization. (B) HEK cells transiently transfected with either GFP and PSTPIP1-myc or dynamin 2-GFP and PSTPIP1-myc were lysed; the coimmunoprecipitations with either mouse IgG control or mouse anti-myc were performed as described in *Materials and Methods*. Immunoblot of the lysates and specific immunoprecipitations are shown. Anti-GFP was used to detect GFP-dynamin 2 and GFP, and anti-PSTPIP1 was used to detect PSTPIP1-myc. All samples were run on the same gel, and the resulting image was adjusted before intervening lanes were removed for clarity. (C) Far Western analysis shows that PSTPIP1 and dynamin 2 directly interact. ^{35}S-labeled PSTPIP was generated from the pcDNA 3.1-PSTPIP1-myc. Exogenously expressed GFP, GFP-dynamin 2, or flag-tagged N-WASP proteins were isolated by immunoprecipitation, resolved by SDS-PAGE, and transferred to nitrocellulose. Membranes were denatured/renatured, blocked, and then incubated with the probe. After an overnight incubation, bound probe was visualized using the STORM PhosphoImager system (left panels). Also shown is the control immunoblot showing amounts of immunoprecipitated proteins (right panels).

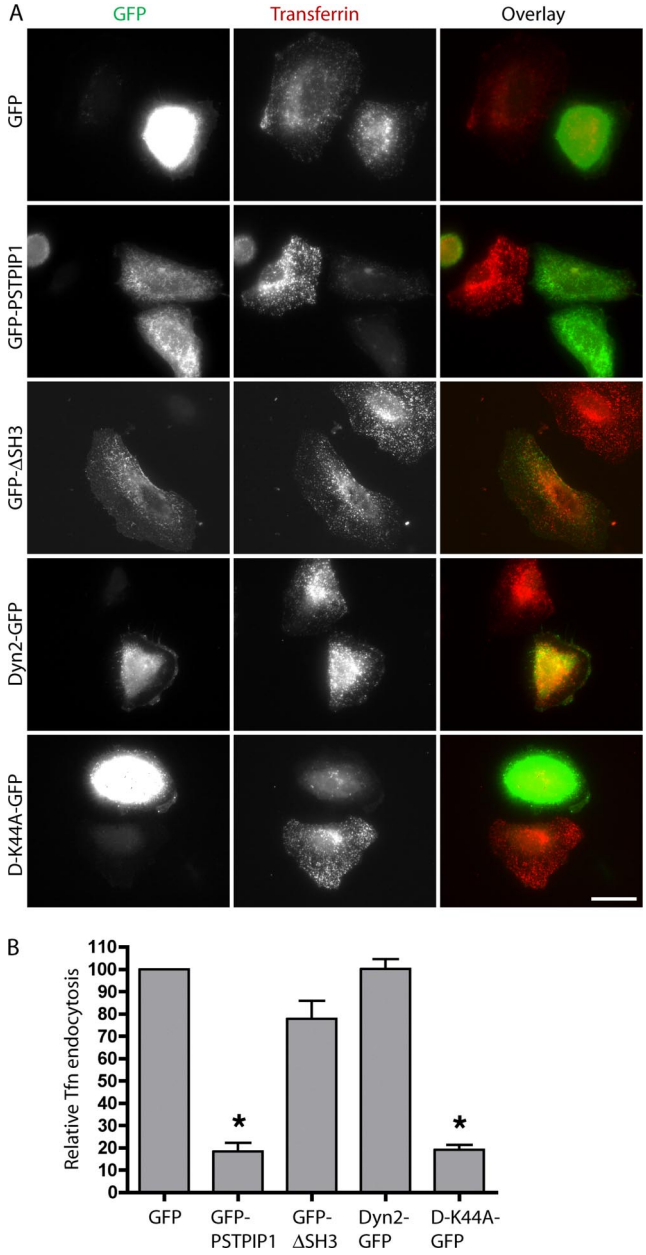

Figure 4. PSTPIP1 expression impairs endocytosis in HeLa cells. (A) HeLa cells were transiently transfected with GFP, GFP-PSTPIP1, GFP-PSTPIP1-ΔSH3 (GFP-ΔSH3), GFP-dynamin 2, or GFP-dynamin 2-K44A (D-K44A-GFP), as indicated. Serum-starved cells were incubated with rhodamine-conjugated transferrin (30 μg/ml) for 10 min and then acid-washed to remove transferrin from the surface. Cells were fixed and imaged. Shown are representative images of GFP expression and rhodamine-transferrin. (B) Quantification of endocytosis. The percent of transfected cells that performed endocytosis of transferrin was determined and expressed relative to GFP control as the mean ± SEM for three independent experiments. Control GFP cells had between 85 and 96% transferrin uptake in each experiment. Significant differences in endocytosis of the raw values between the indicated condition and the GFP-alone cells; *$p < 0.01$, ANOVA, Tukey posttests.

without affecting its dynamics in vitro or in vivo, and can be used to distinguish between dynamic and stable F-actin in vivo. To determine the localization of the utrophin probe in migrating dHL-60 cells, RFP-tagged Utr-CH was transiently

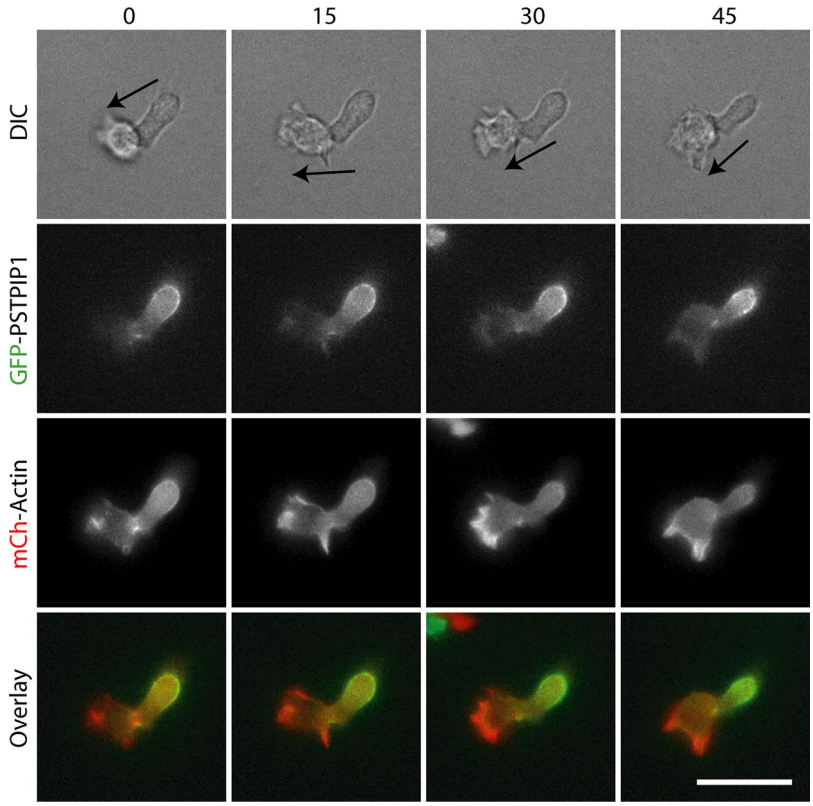

Figure 5. PSTPIP1 does not colocalize with dynamic actin at the leading edge of the cell. Representative DIC and fluorescence images of dHL-60 cells that stably express GFP-PSTPIP1 and transiently express mCherry-actin. Images were taken at 15-s intervals in cells treated with a uniform concentration of 10 nM fMLP. Results are shown for a representative movie from a minimum of three independent experiments. Bar, 10 μm. Arrows show the direction of cell migration.

expressed in wild-type dHL-60 cells, and time-lapse imaging was performed of cells migrating in a uniform concentration of fMLP. Surprisingly, Utr-CH localized to the uropod of migrating dHL-60 cells and was excluded from the leading edge of the cell. To determine if Utr-CH colocalized with GFP-actin, they were both transiently expressed in dHL-60 cells. In general, GFP-actin targeted to areas of active actin polymerization in the pseudopods and did not colocalize with RFP-Utr-CH (Figure 6). These findings suggest that there are different populations of F-actin in dHL-60 cells with highly dynamic actin labeled with GFP-actin at the leading edge of the cell and more stable actin detected by the binding of RFP-Utr-CH at the uropod of the cell.

To determine if PSTPIP1 colocalizes with the more stable F-actin concentrated toward the rear of the cell, dHL-60 cells that stably express GFP-PSTPIP1 were transiently transfected with RFP-Utr-CH. We found that GFP-PSTPIP1 and RFP-Utr-CH colocalized toward the uropod during dHL-60 random migration, suggesting that PSTPIP1 associates with a subpopulation of less dynamic actin within the cell (Figure 7). These findings are consistent with some reports, suggesting that PCH family members may promote actin bundling (Chitu *et al.*, 2005), but also raise intriguing questions about whether or not actin polymerization or dynamics can modulate the targeting of specific adaptor proteins within the cell.

Actin Polymerization, But Not the Microtubule Network, Is Necessary for PSTPIP1 Localization at the Uropod

Previous studies have shown that the microtubule network orients to the rear of the cell during directed cell migration (Eddy *et al.*, 2002). To determine if the microtubule network was necessary for PSTPIP1 targeting to the uropod, dHL-60 cells were treated with colchicine and motility was observed by live fluorescent imaging. In agreement with previous reports (Xu *et al.*, 2005), microtubule disruption did not impair motility, but did inhibit chemotaxis. However, regardless of the direction of migration, GFP-PSTPIP1 localization to the rear of the cell was not affected by microtubule disruption (Figure S2).

To determine if actin polymerization was necessary for PSTPIP1 localization to the uropod, dHL-60 cells that stably express GFP-PSTPIP1 were treated with latrunculin B. High concentrations (2–5 μM) of latrunculin B induced cell rounding and a loss of the polarized distribution of GFP-PSTPIP1 (data not shown). However, we also examined the effects of lower concentrations of latrunculin B that did not immediately induce cell rounding (1 μM). Low-dose latrunculin B impaired the targeting of RFP-Utr-CH to the uropod and resulted in diffuse cytoplasmic distribution, suggesting that this concentration of latrunculin was sufficient to disrupt the actin cytoskeleton (Figure 8A). We also found that in the presence of low-dose latrunculin GFP-PSTPIP1 no longer polarized toward the rear of the cell (Figure 8B). Furthermore, in the presence of latrunculin, the localized puncta that contain PSTPIP1 enlarged and formed into longer tubules that were present both at the front and rear of the cell (Figure 8.mov). Interestingly, we found that inhibition of actomyosin-based contractility with blebbistatin also impaired the localization of PSTPIP1 to the uropod (Figure S3), suggesting that actomyosin-based contractility is necessary for PSTPIP1 targeting to the uropod. In accordance with the previous publications with other PCH family members in adherent cell types (Itoh *et al.*, 2005), these findings suggest that actin polymerization modulates the localization of PSTPIP1 and its association into membrane tubules in neutrophils.

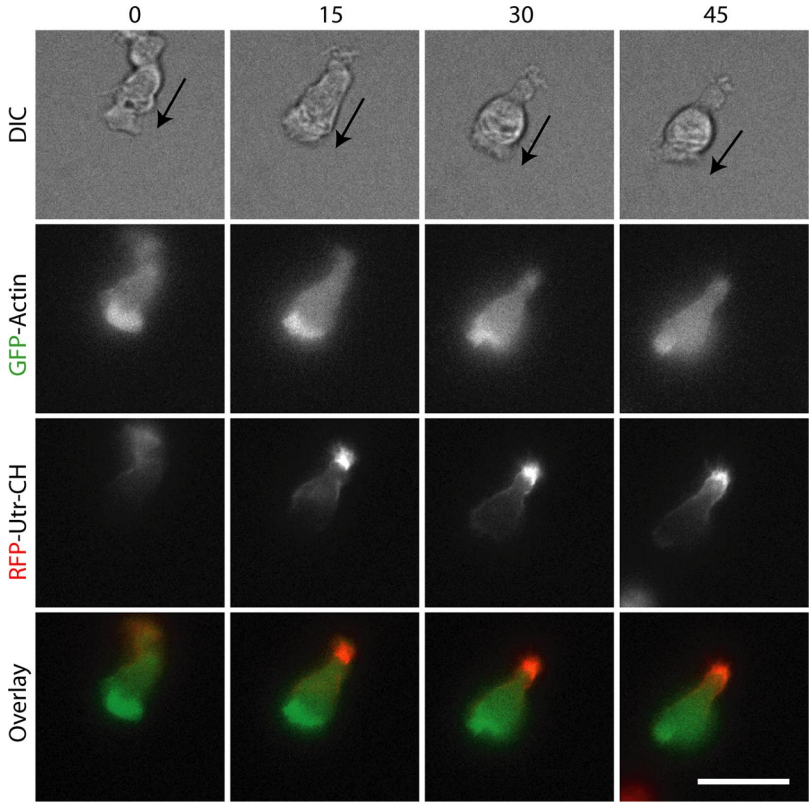

Figure 6. The calponin homology domain from utrophin probes a novel population of actin at the leukocyte uropod. Representative images from dHL-60 cells that transiently express RFP-Utr-CH (red) and GFP-actin (green) and were treated with a uniform concentration of 10 nM fMLP. Images were taken every 15 s as indicated. Results are shown for a representative movie from a minimum of three independent experiments. Bar, 10 μm. Arrows show the direction of cell migration.

To further characterize the role of actin dynamics on the localization of PSTPIP1, we examined the effects of the drug jasplakinolide, which stabilizes actin filaments on the targeting of RFP-Utr-CH and GFP-PSTPIP1 in dHL-60 cells. In the

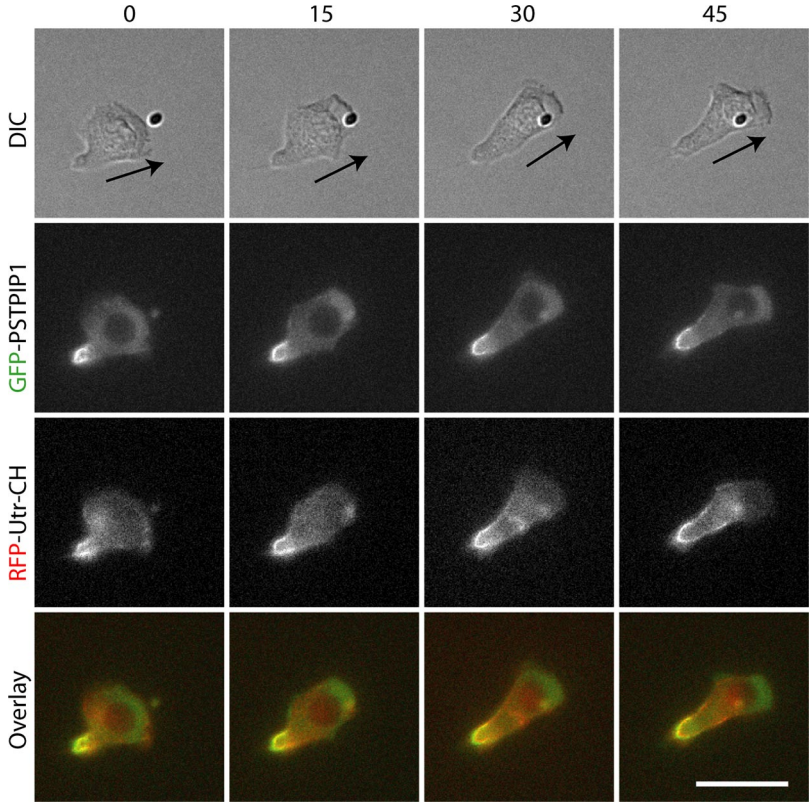

Figure 7. PSTPIP1 colocalizes with the Utr-CH probe at the uropod of dHL-60 cells. Representative DIC and fluorescence images from dHL-60 cells that stably express GFP-PSTPIP1 (green) and transiently express RFP-Utr-CH (red). Cells were treated with a uniform concentration of 10 nM fMLP, and images were taken every 15 s as indicated. Results are shown for a representative movie from a minimum of three independent experiments. Bar, 10 μm. Arrows show the direction of cell migration.

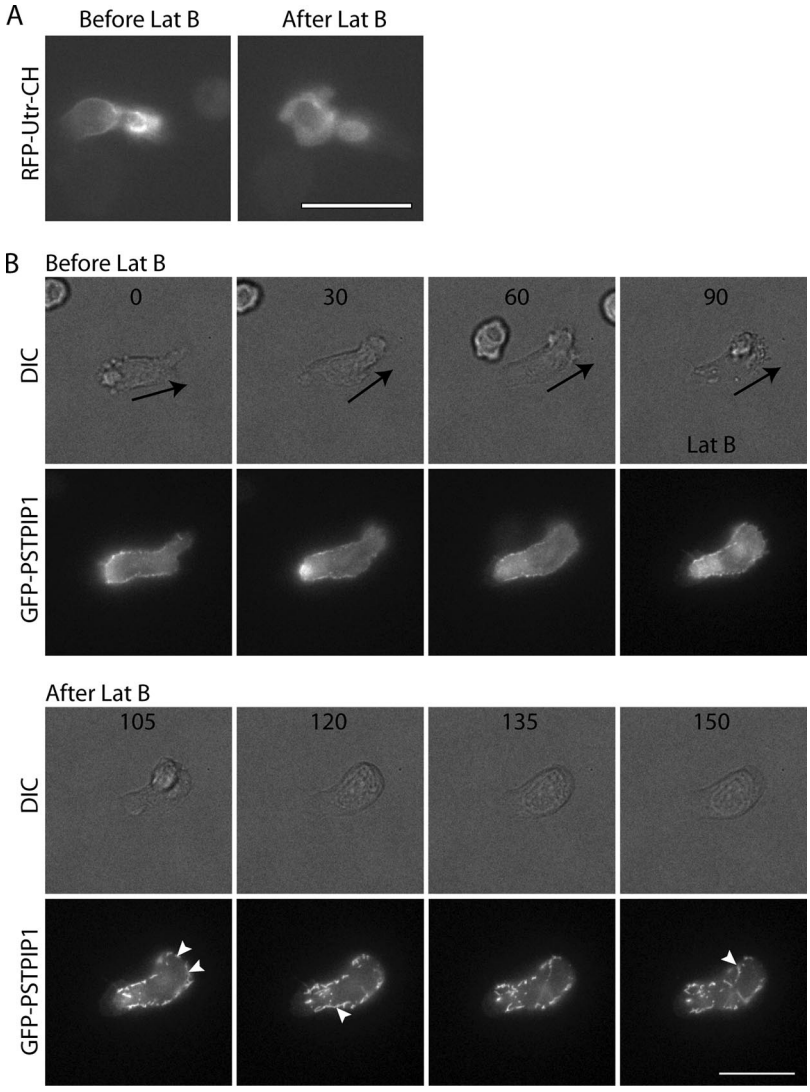

Figure 8. Disruption of the actin cytoskeleton perturbs PSTPIP1 localization at the uropod and induces membrane tubulation. (A) Representative DIC and fluorescence images from dHL-60 cells that transiently express RFP-Utr-CH and were treated with a uniform concentration of 10 nM fMLP. Shown is one frame before addition of latrunculin B (1 μM) and one frame 30 s after addition. (B) Representative DIC and fluorescence images from dHL-60 cells that stably express GFP-PSTPIP1 and were treated with a uniform concentration of 10 nM fMLP. Images were taken every 30 s as indicated and latrunculin B (1 μM) was added at 90 s as indicated. Results are shown for a representative movie from a minimum of three independent experiments. Bar, 10 μm.

presence of fMLP, treatment with jasplakinolide induced the cortical distribution of RFP-Utr-CH along the membrane at both the front and rear of the cell, suggesting that stabilized actin can modulate the targeting of the utrophin probe (Figure 9A). Accordingly, we also found that GFP-PSTPIP1 distribution became more diffuse in a cortical distribution along the membrane in the presence of jasplakinolide (Figure 9B). Taken together, these findings suggest that actin dynamics is an important regulator of PSTPIP1 localization to the uropod during directed cell migration.

DISCUSSION

PCH family members have emerged as important regulators of membrane–cytoskeletal interactions. Here we show that PSTPIP1, a PCH family member expressed in hematopoietic cells, regulates the motility of neutrophil-like cells and is a novel component of the leukocyte uropod where it colocalizes with other uropod components, such as type I PIPKIγ. Furthermore, we show that PSTPIP1 associates with the regulator of endocytosis, dynamin 2, and PSTPIP1 expression impairs transferrin uptake and endocytosis. Collectively, these findings suggest that a complex of proteins, including PSTPIP1, dynamin 2 and PIPKIγ, localize to the uropod of neutrophil-like cells where they may function to regulate endocytosis and membrane trafficking. We also show that PSTPIP1 associates at the rear of neutrophils with a more stable population of F-actin, as detected by the binding of the F-actin probe Utr-CH and that actin polymerization is necessary for the polarized distribution of PSTPIP1 toward the rear of the cell.

Chemoattractant stimulation induces an internal asymmetry of signaling molecules that elicit front-specific actin polymerization and back-specific actomyosin-based contractility. The components at the leading edge of the cell, including PI3K and asymmetric activation of Rac and Cdc42, orchestrate the dynamic assembly and disassembly of actin filaments at the leading edge that mediate forward protrusion. Although progress has been made in defining Rho/ROCK signaling in mediating the backness response, progress in identifying the components of the leukocyte uropod have remained limited. Our recent work identified PIPKIγ as a component of the neutrophil uropod that mediates the localized generation of PtdIns(4,5)P_2 at the rear of the cell during chemotaxis (Lokuta *et al.*, 2007). Here, we show that PSTPIP1 is a novel component of the leukocyte uropod

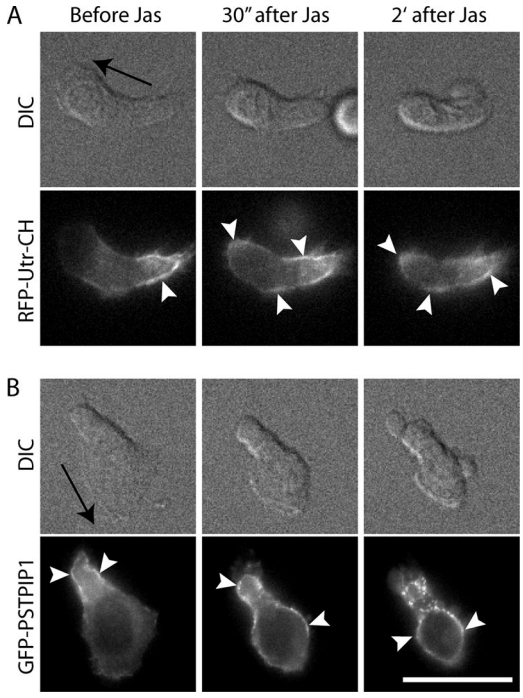

Figure 9. Stabilization of the actin cytoskeleton perturbs PSTPIP1 localization to the uropod. Representative DIC and fluorescence images from dHL-60 cells that transiently express either (A) RFP-Utr-CH or (B) GFP-PSTPIP1 and were treated with a uniform concentration of 10 nM fMLP. Images were taken before addition of jasplakinolide (1 μM) and afterward, with the 30-s and 2-min time points shown. Arrows indicate direction of cell movement. Arrowheads indicate areas of localization. Bar, 10 μm.

where it colocalizes with PIPKIγ. This targeting is in contrast to the localization of the closely related PCH family member, MAYP or PSTPIP2, which is not expressed in neutrophils (Grosse *et al.*, 2006) and targets to areas of membrane protrusion that contain filopodial F-actin in macrophages (Chitu *et al.*, 2005). Because PSTPIP2 is highly homologous to PSTPIP1 but lacks the C-terminal SH3 domain these findings would suggest that the SH3 domain may be important for uropod targeting. To our surprise a truncated form of PSTPIP1 that lacks the SH3 domain also targeted to the uropod of neutrophils and dHL-60 cells, suggesting that interactions with binding proteins through the SH3 domain of PSTPIP1 are not necessary for the targeting of PSTPIP1 to the rear of the cell.

Previous work has shown that PIPKIγ associates with AP-2 to regulate endocytosis (Bairstow *et al.*, 2006). More recent studies report that AP-2 is polarized to the uropod of T-cells with GFP-clathrin and contributes to polarized clathrin-mediated endocytosis (CME) at the rear of T-cells during directed cell migration (Samaniego *et al.*, 2007). We have now identified several novel components of the leukocyte uropod including dynamin 2 and PSTPIP1. The association of PSTPIP1 with dynamin and its colocalization with PIPKIγ at the rear of the cell suggest that PSTPIP1/dynamin and PIPKI may represent a novel complex at the cell rear that regulates endocytosis and membrane trafficking during neutrophil migration. Because endocytosis and recycling of cell surface receptors have been implicated in neutrophil rear release (Pierini *et al.*, 2000), and more recently, at the uropod of T-cells (Samaniego *et al.*, 2007), it is intriguing to speculate that PSTPIP1 may be involved in localized membrane trafficking and endocytosis at the rear of neutrophils. Although we did not see any evidence that modulation of PSTPIP1 expression affected global internalization of chemoattractant receptors after stimulation with chemoattractant (data not shown), it does not exclude a role for PSTPIP1 in localized trafficking of membrane or surface receptors at the rear of the cell. Furthermore, evidence implicates a role for asymmetric membrane recycling with exocytosis at the leading edge of the cell and endocytosis at the rear of the cell in polarized cell movement (Bretscher, 1996). Our findings suggest that specific PCH proteins, notably PSTPIP1 in leukocytes, may contribute to this asymmetric membrane dynamics by regulating actomyosin assembly and membrane dynamics at the uropod of the cell during migration.

A critical determinant of PCH family protein function is their association with and regulation by the actin cytoskeleton. PSTPIP1 binds to WASP and mediates its interaction with PTP-PEST, which dephosphorylates and negatively regulates WASP activity (Cote *et al.*, 2002; Torres and Rosen, 2003). Because WASP is found both at the front and trailing edge of some cells during directed cell migration (Myers *et al.*, 2005), it is possible that PSTPIP1 may function to restrict WASP activity and actin dynamics to the front of the cell during polarized cell migration. Accordingly, ectopic expression of PSTPIP1 may impair leukocyte chemotaxis by negatively regulating WASP activity. It is also possible that PSTPIP1 may directly modulate actin functions at the rear by affecting actin bundling since the closely related family member PSTPIP2 has been shown to promote actin bundling (Chitu *et al.*, 2005). In agreement with this possibility is the finding that expression of PSTPIP1-ΔSH3 impairs migration, indicating that PSTPIP1 may affect chemotaxis independent of its role in regulating endocytosis. Additionally, we found that PSTPIP1 associated with a novel subpopulation of cortical F-actin at the dHL-60 uropod that is detected by the probe Utr-CH.

Previous studies have indicated that at the leading edge of neutrophils, actin is highly dynamic and can be monitored by expression of GFP-actin or by the expression of specific actin-binding proteins that associate with dynamic actin, such as Arp 3-GFP (Weiner *et al.*, 1999). To date, there have been no probes that have distinguished between the different populations of F-actin at the front and rear of leukocytes during directed migration. We found that the calponin homology domain of utrophin (Utr-CH) represents a novel probe of the leukocyte uropod. Utr-CH has previously been reported to bind less dynamic actin in *Xenopus* oocytes, where it associates with actin in areas of membrane protrusion (Burkel *et al.*, 2007). We have also found that it localizes to areas of membrane protrusion in fibroblast cells (data not shown). These differences likely reflect the highly dynamic nature of actin at leading edge of leukocytes as compared with mesenchymal cells. We now show that the Utr-CH labels a specific population of actin that is found at the leukocyte uropod and does not colocalize with Arp3-GFP (data not shown) or GFP-actin at the leading edge of the cell. This observation will pave the way for future studies that address the nature of uropodal actin and its dynamics during directed migration. For the purposes of this study, however, it is intriguing that PSTPIP1 specifically colocalized with this population of actin at the uropod of dHL-60 cells.

These studies raise the interesting question regarding the potential role of actin dynamics in polarizing specific proteins to the leukocyte uropod. Actin polymerization is necessary for the initiation and establishment of cell polarity during directed migration through amplifying signaling responses at the leading edge of the cell via a positive feedback

mechanism. A simple way to polarize proteins away from the leading edge of the cell would be their exclusion from regions of active actin polymerization. The specific targeting of Utr-CH probe to the uropod is an example of how an actin-binding protein may be excluded form the leading edge dynamic actin. It is possible that similar mechanisms may contribute to the polarized distribution of PSTPIP1 and the endocytic machinery away from the leading edge of the cell. Amplification of this response may occur through the down-regulation of actin-based protrusions and signaling through regulated endocytosis and the inhibition of proteins such as WASP. Consistent with this hypothesis, we found that disruption of the actin cytoskeleton with low-dose latrunculin induced the translocation of PSTPIP1 into membrane tubules both at the rear and front of the cell and jasplakinolide treatment that stabilizes actin also impaired PSTPIP1 targeting to the uropod, suggesting that actin dynamics may be restricting the localization and functions of PSTPIP1 and its associated proteins.

Neutrophil trafficking and regulated motility are important for a variety of diseases, including chronic inflammatory diseases. Specifically, mutations in PSTPIP1 are associated with the autoinflammatory disorder PAPA syndrome, characterized by neutrophil infiltration into tissues including the skin and joints in the absence of infection. The involvement of PSTPIP1 in neutrophil motility suggests that abnormalities in leukocyte trafficking induced by disease mutations may contribute to the disease phenotype. Consistent with a role of leukocyte motility in the pathogenesis of autoinflammatory diseases, we have previously shown that the migration of neutrophils from patients with other autoinflammatory diseases, such as NOMID, are impaired (Huttenlocher *et al.*, 1995; Lokuta *et al.*, 2005). Because PSTPIP1 is mutated in the autoinflammatory disease PAPA syndrome, we investigated the function of the disease-causing mutant PSTPIP1 protein in our system. The mutant protein (either GFP-PSTPIP1-E250Q or GFP-PSTPIP1-A230T) localized normally toward the rear of the dHL-60 cell and expression impaired dHL-60 cell migration by transwell assay to the same extent as the wild-type protein (data not shown). These findings suggest a complicated relationship between PSTPIP1 activity and neutrophil migration and how these mechanisms may contribute to the pathogenesis of PAPA syndrome. Dissecting these pathways and interactions will be the challenge for future investigations.

In conclusion, we have identified a hematopoietic PCH family member PSTPIP1, which targets to the leukocyte uropod and regulates directed cell migration. Furthermore, our findings indicate that PSTPIP1 colocalizes with a distinct subpopulation of F-actin at the leukocyte uropod that is detected by the F-actin probe Utr-CH and that its localization is regulated by actin polymerization. A challenge for future studies will be to understand the feedback mechanisms by which dynamic actin polymerization may contribute to the asymmetric targeting of PSTPIP1 and other proteins that regulate membrane cytoskeletal dynamics and contribute to the amplification of cell polarity during directed cell migration.

ACKNOWLEDGMENTS

We gratefully acknowledge Jun Zhu for the cloning of pmCherry-PSTPIP1, Christa Cortesio for the cloning of pcDNA3.1 flag-N-WASP; Daniel Kastner (National Institutes of Health, Bethesda, MD) for the pcDNA3.1-PSTPIP1-myc construct; Mark McNiven (Mayo Clinic, Rochester, MN) for the GFP-dynamin2 constructs; Orion Weiner (University of California, San Francisco) for the Arp3-GFP construct; Roger Tsien (University of California, San Diego) for the mCherry vector; Gerald Marriott (University of Wisconsin, Madison) for the mCherry-actin construct; William Bement (University of Wisconsin, Madison) for the RFP-Utr-CH construct; Kevin Walters, Christa Cortesio, and Ashley Doan Conrad for critical reading of the manuscript; and Kathy Schell, Joel Puchalski, and Dagna Sheerar for their expertise at the Flow Cytometry Facility (University of Wisconsin, Madison). We thank William Bement, Santos Franco, and Benjamin Perrin for helpful discussions. This work was supported by National Institutes of Health Grants R01 GM074827 (A.H.) and T32 GM07215 (K.M.C.).

REFERENCES

Babior, B. M., and Matzner, Y. (1997). The familial Mediterranean fever gene—cloned at last. N. Engl. J. Med. *337*, 1548–1549.

Badour, K., Zhang, J., Shi, F., McGavin, M. K., Rampersad, V., Hardy, L. A., Field, D., and Siminovitch, K. A. (2003). The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. Immunity *18*, 141–154.

Bairstow, S. F., Ling, K., Su, X., Firestone, A. J., Carbonara, C., and Anderson, R. A. (2006). Type Igamma661 phosphatidylinositol phosphate kinase directly interacts with AP2 and regulates endocytosis. J. Biol. Chem. *281*, 20632–20642.

Bennin, D. A., Don, A. S., Brake, T., McKenzie, J. L., Rosenbaum, H., Ortiz, L., DePaoli-Roach, A. A., and Horne, M. C. (2002). Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B′ subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. J. Biol. Chem. *277*, 27449–27467.

Bretscher, M. S. (1996). Moving membrane up to the front of migrating cells. Cell *85*, 465–467.

Burkel, B. M., von Dassow, G., and Bement, W. M. (2007). Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin. Cell Motil. Cytoskelet. *64*, 822–832.

Cao, H., Garcia, F., and McNiven, M. A. (1998). Differential distribution of dynamin isoforms in mammalian cells. Mol. Biol. Cell *9*, 2595–2609.

Chitu, V., Pixley, F. J., Macaluso, F., Larson, D. R., Condeelis, J., Yeung, Y. G., and Stanley, E. R. (2005). The PCH family member MAYP/PSTPIP2 directly regulates F-actin bundling and enhances filopodia formation and motility in macrophages. Mol. Biol. Cell *16*, 2947–2959.

Chitu, V., and Stanley, E. R. (2007). Pombe Cdc15 homology (PCH) proteins: coordinators of membrane-cytoskeletal interactions. Trends Cell Biol. *17*, 145–156.

Church, L. D., Cook, G. P., and McDermott, M. F. (2008). Primer: inflammasomes and interleukin 1beta in inflammatory disorders. Nat. Clin. Pract. Rheumatol. *4*, 34–42.

Cote, J. F., Chung, P. L., Theberge, J. F., Halle, M., Spencer, S., Lasky, L. A., and Tremblay, M. L. (2002). PSTPIP is a substrate of PTP-PEST and serves as a scaffold guiding PTP-PEST toward a specific dephosphorylation of WASP. J. Biol. Chem. *277*, 2973–2986.

Devreotes, P., and Janetopoulos, C. (2003). Eukaryotic chemotaxis: distinctions between directional sensing and polarization. J. Biol. Chem. *278*, 20445–20448.

Eddy, R. J., Pierini, L. M., Matsumura, F., and Maxfield, F. R. (2000). Ca^{2+}-dependent myosin II activation is required for uropod retraction during neutrophil migration. J. Cell Sci. *113*(Pt 7), 1287–1298.

Eddy, R. J., Pierini, L. M., and Maxfield, F. R. (2002). Microtubule asymmetry during neutrophil polarization and migration. Mol. Biol. Cell *13*, 4470–4483.

Engqvist-Goldstein, A. E., Zhang, C. X., Carreno, S., Barroso, C., Heuser, J. E., and Drubin, D. G. (2004). RNAi-mediated Hip1R silencing results in stable association between the endocytic machinery and the actin assembly machinery. Mol. Biol. Cell *15*, 1666–1679.

Grosse, J. *et al.* (2006). Mutation of mouse Mayp/Pstpip2 causes a macrophage autoinflammatory disease. Blood *107*, 3350–3358.

Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. J. Cell Biol. *122*, 565–578.

Hull, K. M., Shoham, N., Chae, J. J., Aksentijevich, I., and Kastner, D. L. (2003). The expanding spectrum of systemic autoinflammatory disorders and their rheumatic manifestations. Curr. Opin. Rheumatol. *15*, 61–69.

Huttenlocher, A., Frieden, I. J., and Emery, H. (1995). Neonatal onset multisystem inflammatory disease. J. Rheumatol. *22*, 1171–1173.

Huttenlocher, A., Ginsberg, M. H., and Horwitz, A. F. (1996). Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. J. Cell Biol. *134*, 1551–1562.

Itoh, T., Erdmann, K. S., Roux, A., Habermann, B., Werner, H., and De Camilli, P. (2005). Dynamin and the actin cytoskeleton cooperatively regulate

plasma membrane invagination by BAR and F-BAR proteins. Dev. Cell *9*, 791–804.

Lacalle, R. A., Peregil, R. M., Albar, J. P., Merino, E., Martinez, A. C., Merida, I., and Manes, S. (2007). Type I phosphatidylinositol 4-phosphate 5-kinase controls neutrophil polarity and directional movement. J. Cell Biol. *179*, 1539–1553.

Lokuta, M. A., Cooper, K. M., Aksentijevich, I., Kastner, D. L., and Huttenlocher, A. (2005). Neutrophil chemotaxis in a patient with neonatal-onset multisystem inflammatory disease and Muckle-Wells syndrome. Ann. Allergy Asthma Immunol. *95*, 394–399.

Lokuta, M. A., Nuzzi, P. A., and Huttenlocher, A. (2003). Calpain regulates neutrophil chemotaxis. Proc. Natl. Acad. Sci. USA *100*, 4006–4011.

Lokuta, M. A., Senetar, M. A., Bennin, D. A., Nuzzi, P. A., Chan, K. T., Ott, V. L., and Huttenlocher, A. (2007). Type Iγ PIP kinase is a novel uropod component that regulates rear retraction during neutrophil chemotaxis. Mol. Biol. Cell *18*, 5069–5080.

Myers, S. A., Han, J. W., Lee, Y., Firtel, R. A., and Chung, C. Y. (2005). A *Dictyostelium* homologue of WASP is required for polarized F-actin assembly during chemotaxis. Mol. Biol. Cell *16*, 2191–2206.

Neven, B. *et al.* (2004). Molecular basis of the spectral expression of CIAS1 mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU. Blood *103*, 2809–2815.

Niggli, V. (1999). Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration. FEBS Lett. *445*, 69–72.

Niggli, V. (2003a). Microtubule-disruption-induced and chemotactic-peptide-induced migration of human neutrophils: implications for differential sets of signalling pathways. J. Cell Sci. *116*, 813–822.

Niggli, V. (2003b). Signaling to migration in neutrophils: importance of localized pathways. Int. J. Biochem. Cell Biol. *35*, 1619–1638.

Nuzzi, P. A., Lokuta, M. A., and Huttenlocher, A. (2007). Analysis of neutrophil chemotaxis. Methods Mol. Biol. *370*, 23–36.

Parent, C. A. (2004). Making all the right moves: chemotaxis in neutrophils and *Dictyostelium*. Curr. Opin. Cell Biol. *16*, 4–13.

Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., and Devreotes, P. N. (1998). G protein signaling events are activated at the leading edge of chemotactic cells. Cell *95*, 81–91.

Pierini, L. M., Lawson, M. A., Eddy, R. J., Hendey, B., and Maxfield, F. R. (2000). Oriented endocytic recycling of alpha5beta1 in motile neutrophils. Blood *95*, 2471–2480.

Ruoslahti, E., Hayman, E. G., Pierschbacher, M., and Engvall, E. (1982). Fibronectin: purification, immunochemical properties, and biological activities. Methods Enzymol. *82*(Pt A), 803–831.

Samaniego, R., Sanchez-Martin, L., Estecha, A., and Sanchez-Mateos, P. (2007). Rho/ROCK and myosin II control the polarized distribution of endocytic clathrin structures at the uropod of moving T lymphocytes. J. Cell Sci. *120*, 3534–3543.

Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science *287*, 1037–1040.

Servant, G., Weiner, O. D., Neptune, E. R., Sedat, J. W., and Bourne, H. R. (1999). Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. Mol. Biol. Cell *10*, 1163–1178.

Shoham, N. G., Centola, M., Mansfield, E., Hull, K. M., Wood, G., Wise, C. A., and Kastner, D. L. (2003). Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. Proc. Natl. Acad. Sci. USA *100*, 13501–13506.

Spencer, S., Dowbenko, D., Cheng, J., Li, W., Brush, J., Utzig, S., Simanis, V., and Lasky, L. A. (1997). PSTPIP: a tyrosine phosphorylated cleavage furrow-associated protein that is a substrate for a PEST tyrosine phosphatase. J. Cell Biol. *138*, 845–860.

Torres, E., and Rosen, M. K. (2003). Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. Mol. Cell *11*, 1215–1227.

Tsujita, K., Suetsugu, S., Sasaki, N., Furutani, M., Oikawa, T., and Takenawa, T. (2006). Coordination between the actin cytoskeleton and membrane deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. J. Cell Biol. *172*, 269–279.

Weiner, O. D., Marganski, W. A., Wu, L. F., Altschuler, S. J., and Kirschner, M. W. (2007). An actin-based wave generator organizes cell motility. PLoS Biol. *5*, e221.

Weiner, O. D., Servant, G., Welch, M. D., Mitchison, T. J., Sedat, J. W., and Bourne, H. R. (1999). Spatial control of actin polymerization during neutrophil chemotaxis. Nat. Cell Biol. *1*, 75–81.

Wise, C. A., Gillum, J. D., Seidman, C. E., Lindor, N. M., Veile, R., Bashiardes, S., and Lovett, M. (2002). Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder. Hum. Mol. Genet. *11*, 961–969.

Xu, J., Wang, F., Van Keymeulen, A., Rentel, M., and Bourne, H. R. (2005). Neutrophil microtubules suppress polarity and enhance directional migration. Proc. Natl. Acad. Sci. USA *102*, 6884–6889.