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A novel role for 14-kDa phosphohistidine phosphatase in lamellipodia formation

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ABSTRACT

Cell migration involves dynamic regulation of the actin cytoskeleton, which exhibits rapid actin polymerization at the leading edge of migrating cells. This process relies on regulated recruitment of actin nucleators and actin-binding proteins to the leading edge to polymerize new actin filaments. Many of these proteins have been identified, including the actin-related protein (Arp) 2/3 complex, which has emerged as the core player in the initiation of actin polymerization. However, the functional coordination of these proteins is unclear. Previously, we have demonstrated that the 14-kDa phosphohistidine phosphatase (PHP14) is involved in cell migration regulation and affects actin cytoskeleton reorganization. Here, we show that PHP14 may regulate actin remodeling directly and play an important role in dynamic regulation of the actin cytoskeleton. We observed a colocalization of PHP14 with Arp3 and F-actin at the leading edge of migrating cells. Moreover, PHP14 was recruited to the actin remodeling sites in parallel with Arp3 during lamellipodia formation. Furthermore, PHP14 knockdown impaired Arp3 localization at the leading edge of lamellipodia, as well as lamellipodia formation. Most importantly, we found that PHP14 was a novel F-actin-binding protein, displaying an Arp2/3-dependent localization to the leading edge. Collectively, our results indicated a crucial role for PHP14 in the dynamic regulation of the actin cytoskeleton and cell migration.

Introduction

The 14-kDa phosphohistidine phosphatase (PHP14), also known as PHPT1, is the first histidine phosphatase protein identified in vertebrates and is similar to the janus proteins of Drosophila.[1,2] It is broadly expressed in most tissues, and abundantly in the heart and skeletal muscle.[3] Many substrates have been discovered for this phosphatase, such as ATP-citrate lyase,[4] the β subunit of G proteins[5] and transient receptor potential-vanilloid-5 (TRPV5),[6] which mediate a multitude of cellular and biological functions. In previous studies, we have demonstrated that PHP14 played a significant role in lung cancer cell migration, invasion and metastasis, which might involve altering the actin cytoskeleton.[7] However, the mechanism by which PHP14 regulates cell migration and the relationship of PHP14 with the actin cytoskeleton still remains unknown.

Cell migration is a key step in many physiological and pathological processes, such as wound repair, tissue regeneration and metastasis.[8] The actin cytoskeleton

plays a pivotal role in cell migration. A variety of distinct actin cytoskeleton structures mediate cell migration, including lamellipodia, filopodia and possibly blebs formed at the leading edge of motile cells.[9] The lamellipodium is considered the best characterized motile organelle; its formation relies on regulated recruitment of molecular scaffolds to its tip.[10] Specifically, the Arp2/3 complex is decisive for actin filament nucleation in the lamellipodium, but activation of the Arp2/3 complex needs nucleation promoting factors, such as Wiskott-Aldrich syndrome family protein (WAVE).[11,12] Therefore, the regulation of the Arp2/3 complex, and thus lamellipodia formation, is very important for cell migration.

In this study, we investigated the possible mechanism of PHP14 for regulating cell migration. First, we showed that PHP14 colocalized with Arp3 at the leading edge of migrating cells. Second, we demonstrated that PHP14 knockdown impaired Arp3 localization and lamellipodia formation. Most importantly, we found that PHP14 is a

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novel F-actin-binding protein in vitro, and displays an Arp2/3-dependent localization to the leading edge.

Results

PHP14 localizes to the leading edge of lamellipodia

Previously, we have reported that PHP14 regulated cell migration and may be involved in actin cytoskeleton reorganization,[7] but the precise role of PHP14 plays in cell migration is yet to be addressed. To this end, we investigated in different cell lines the distribution of endogenous PHP14 and its relationship with F-actin and Arp3, a marker of the leading edge sites of lamellipodia. Interestingly, we observed co-staining of PHP14, Arp3 and F-actin at the leading edge of lamellipodia in all 4 cell lines tested (Fig. 1). Lamellipodia are essential for cell motility; their formation relies on regulated recruitment of

molecular scaffolds, e.g., Arp3, to their tips to organize actin filaments into lamella networks and bundled arrays. According to our results, a pool of PHP14 colocalized with Arp3 at the leading edge of lamellipodia, indicating its possible role in lamellipodia formation.

PHP14 is recruited to the actin remodeling sites and the wound-healing front

To elucidate the relationship of PHP14 with lamellipodia formation, we investigated the PHP14 localization change during new lamellipodia formation. We used platelet-derived growth factor (PDGF) to induce lamellipodia formation, as PDGF has clearly been shown to modulate the formation of lamellipodia.[13] Interestingly, the hepatocarcinoma cell line, HuH-7, barely had stress fibers and lamellipodia on glass slides under normal
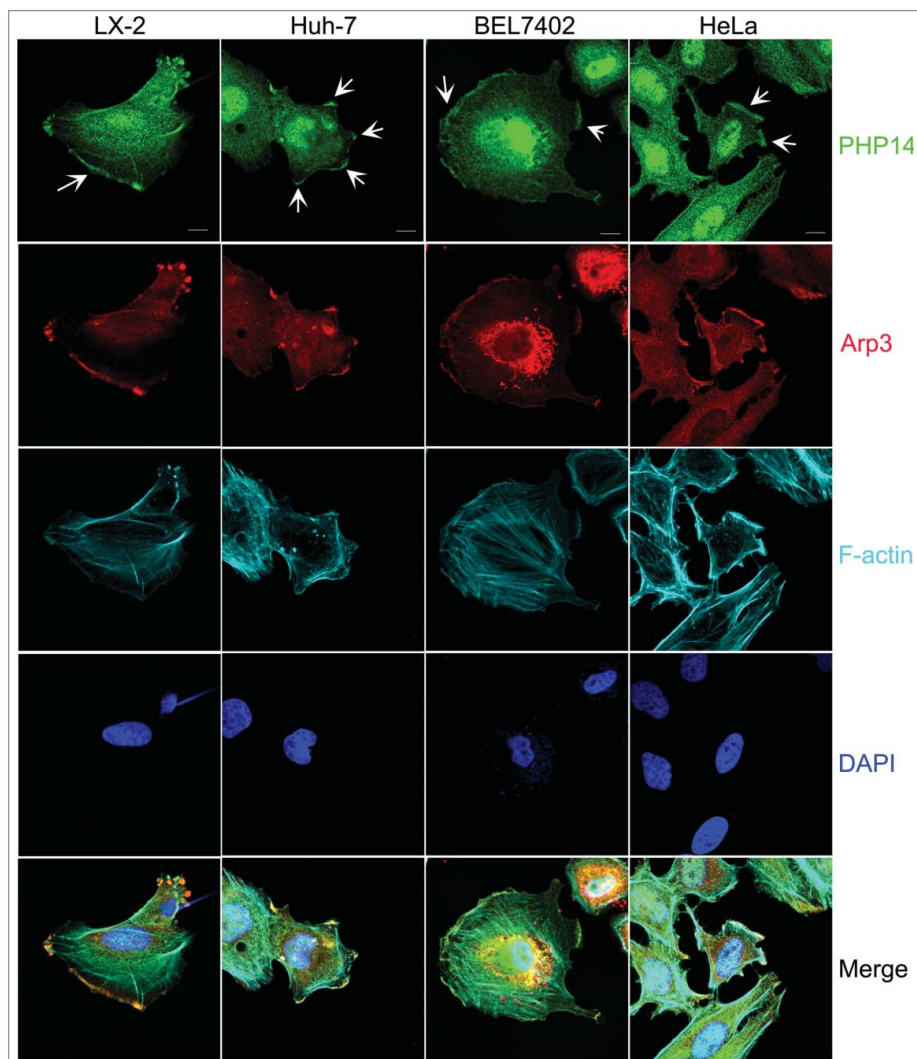
Figure 1. PHP14 colocalizes with Arp3 and F-actin at the cell leading edge in different cell types. LX-2, HuH-7, BEL7402 and HeLa cells were added to coverslips. After cell attachment and spreading, coverslips were fixed and stained with anti-PHP14 and anti-Arp3 antibody, F-actin was stained using rhodamine conjugated phalloidin. Representative images for each cell type are shown. The colocalization of PHP14 and Arp3 at the leading edge of lamellipodia was indicated by arrows. Scale bar 10 μm.

culture conditions, and PHP14 and Arp3 were diffusely distributed in the cells (upper panels of Fig. S1A). In contrast, stress fibers formed and new lamellipodia were induced after PDGF was added, and PHP14 and Arp3 were recruited to the lamellipodia sites (lower panels of Fig. S1A). Therefore, we used HuH-7 cells to monitor the localization of PHP14 in lamellipodia over time by immunofluorescence microscopy. As shown in Fig. 2A, we observed a rapid recruitment of PHP14 and Arp3 to PDGF-induced membrane protrusion sites.

The wound-healing assay is a widely used method for evaluating cell migration in vitro. The cells at the front of the wound moved in and filled the area of the "wounded" region over time. Similarly to our previous studies,[7] the cell motility decreased in PHP14-knockdown LX-2 cells (Fig. S1B and S1C), and this was not caused by different proliferation rates among the cell lines either (Fig. S1D). Furthermore, we detected recruitment of PHP14 to the wound-healing front by immunofluorescence staining (Fig. 2B). Taken together, these data suggest that PHP14 has an active role at leading edge sites during cell migration.
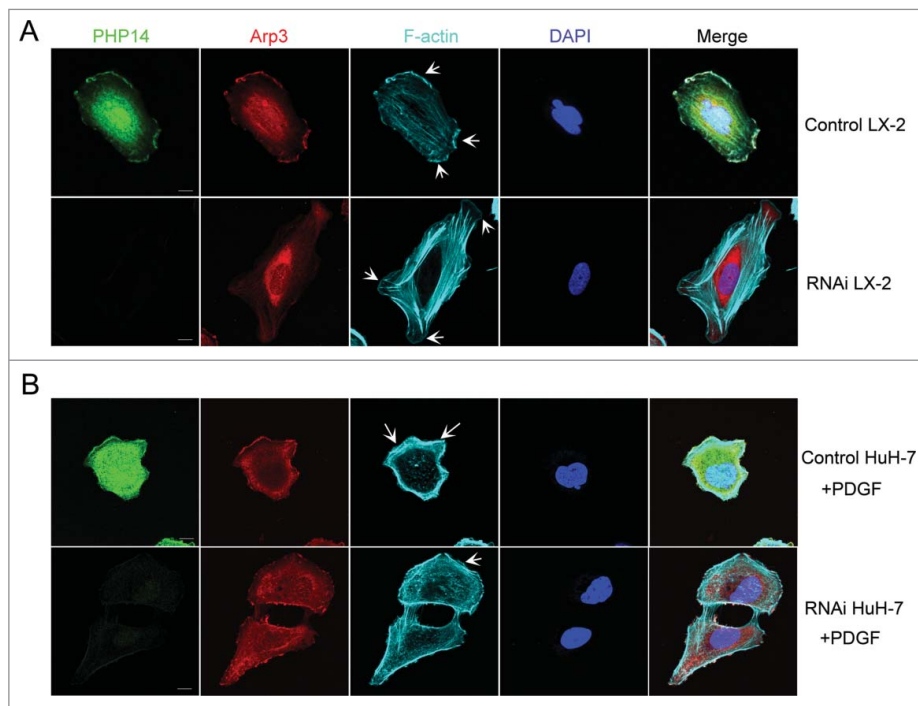
PHP14 knockdown influences Arp3 localization and impairs lamellipodia formation

We next investigated the effect of PHP14 knockdown on lamellipodia formation. PHP14-knockdown LX-2 cells displayed flat morphology with protrusive edges, whereas the control LX-2 cells had smooth lamellipodia-like edges (Fig. S2). Therefore, we conducted F-actin and Arp3 staining to examine the lamellipodia changes in PHP14-knockdown LX-2 cells. The control LX-2 cells exhibited typical lamellipodia, in which Arp3 localized to the leading edge of lamellipodia, while Arp3-staining was absent from the tip of the actin bundles (protrusive edges) in PHP14-knockdown LX-2 cells, and only diffused cytoplasmic staining was observed (Fig. 3A).

To further investigate the effect of PHP14 knockdown on lamellipodia formation, we evaluated the PDGF-induced lamellipodia formation after PHP14 knockdown compared with the control HuH-7 cells. As shown in Fig. 3B, PHP14 knockdown dramatically inhibited the PDGF-induced lamellipodia formation in HuH-7 cells.
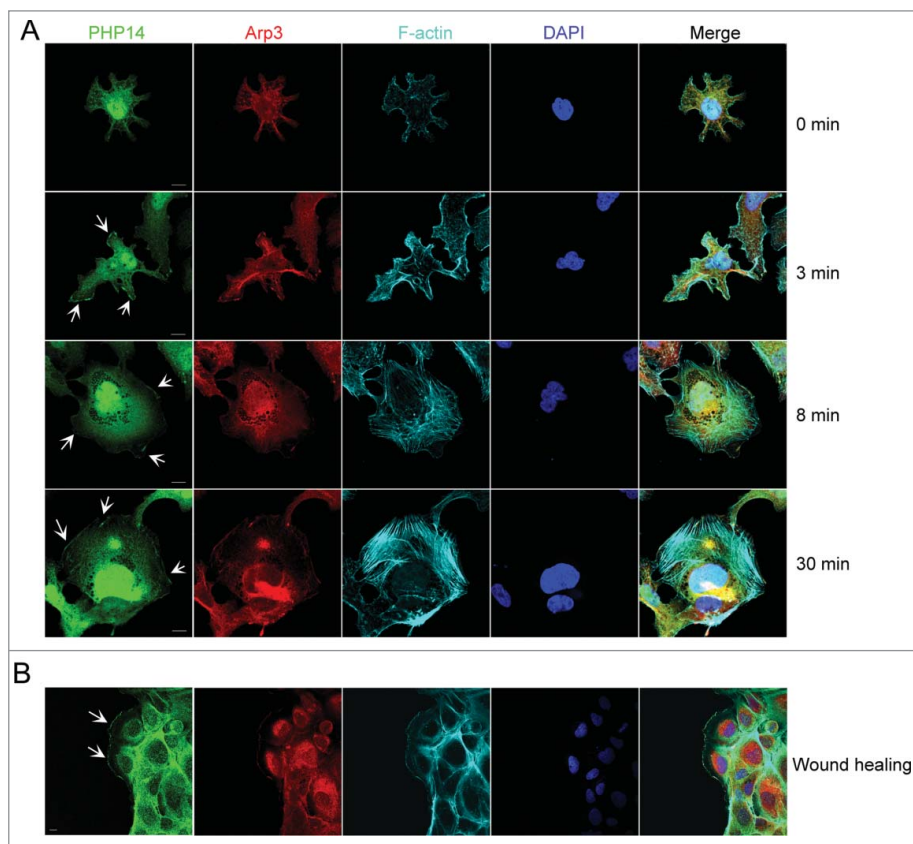
Figure 2. PHP14 is recruited to sites of actin remodelling. (A) Huh-7 cells were treated with PDGF-BB (10 ng/mL) and stained for PHP14, Arp3 and F-actin at different time point. The recruitment of PHP14 in parallel with Arp3 to the PDGF-induced F-actin positive membrane protrusions (indicated by arrows) was observed as early as 3 min after PDGF treatment. Scale bar 10 μm. (B) LX-2 cells were wounded, fixed after 3 hours, and stained for PHP14, Arp3 and F-actin. PHP14 was recruited to the wound healing front (indicated by arrows) immediately after wounding. Scale bar 10 μm.

Figure 3. The effect of PHP14 knockdown on Arp3 localization and lamellipodia formation. (A) LX-2 cells were transfected with either control shRNA or shRNA targeted to PHP14 for 48 hours, and stained for PHP14, Arp3 and F-actin. The staining of Arp3 was absent from the tip of the actin bundles (protrusive edges, indicated by arrows) in PHP14 knockdown LX-2 cells, and only diffuse cytoplasmic staining was observed (lower panels). Scale bar 10 μm. (B) Huh-7 cells were transfected with either control shRNA or shRNA targeted to PHP14 for 48 hours, after 3 min treatment of PDGF-BB (10 ng/mL), cells were stained for PHP14, Arp3 and F-actin. PDGF treatment induced lamellipodia formation in control cells (indicated by arrows), while the protrusive edges were obviously inhibited in PHP14 knockdown Huh-7 cells. Scale bar 10 μm.

PHP14 displays an Arp2/3-dependent localization to the leading edge and binds to the actin cytoskeleton in vitro

Because PHP14 and Arp3 translocated to the leading edge in parallel during lamellipodia formation and colocalized in this region (Fig. 2), we analyzed the possible association between PHP14 and Arp3. As shown in Fig. 4A, PHP14 knockdown suppressed the PDGF-induced Arp3 recruitment to the leading edge, while PHP14-expressing cells displayed PHP14 and Arp3 colocalization in the leading edge. Moreover, PHP14 localization at the leading edge seemed to depend on Arp2/3, because PHP14 gradually disappeared from the leading edge after addition of the Arp2/3 inhibitor, CK-666 (150 μM; Fig. 4B), and its disappearance was also in parallel with Arp3 (Fig. S3A). However, PHP14 did not appear to interact with Arp3, as we did not detect a PHP14-Arp3 interaction by a coimmunoprecipitation assay, even PDGF added (Fig. S3B). Therefore, we speculate that PHP14 binds F-actin to facilitate Arp2/3 forming lamellipodia. In fact, we detected a direct interaction between PHP14 and F-actin in vitro by a high-speed actin co-sedimentation assay (Fig. 4C). Furthermore,

besides PHP14 colocalized with F-actin at the site of the leading edge of lamellipodia in migrating cells, PHP14 colocalized with F-actin fibers when cells reached 100% confluence (Fig. 4D). These results suggest that PHP14 played a role in cell migration, possibly through directly remodeling the actin cytoskeleton.

Discussion

The actin cytoskeleton plays a pivotal role in cell migration. Actin nucleators and actin-binding proteins are distributed at the leading edge, to polymerize new actin filaments or to serve as scaffolds to recruit other proteins necessary for cell migration.[14] In our previous studies, we have found that PHP14 may be involved in actin cytoskeleton reorganization and regulate cell migration.[7] Here, we provided new insight into the function of PHP14 in cell migration.

First, we revealed that PHP14 was localized to the sites of the leading edge of migrating cells and the wound front in a wound-healing assay. Migrating cells form lamellipodia by reorganizing the actin cytoskeleton at the leading edge. This reorganization of the actin
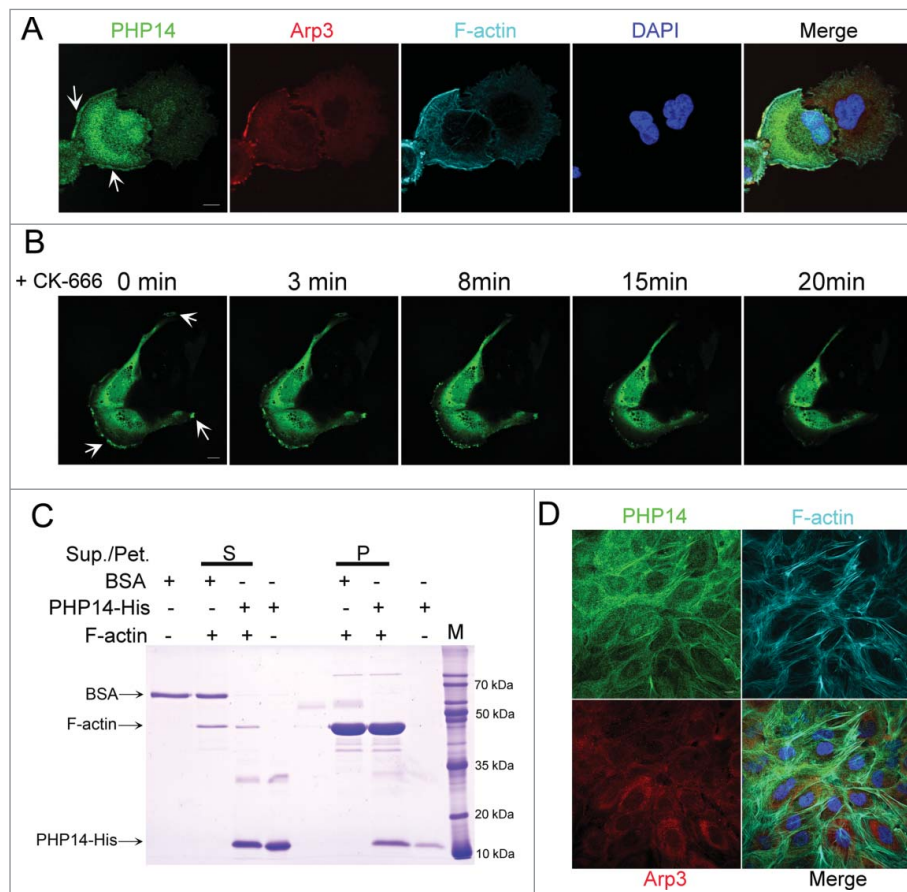
Figure 4. PHP14 displays a Arp2/3-dependent localization to the leading edge and binds to the actin cytoskeleton in vitro. (A) LX-2 cells were transfected with either control shRNA or shRNA targeted to PHP14 for 48 hours, after 3 min treatment of PDGF-BB (10 ng/mL), cells were stained for PHP14, Arp3 and F-actin. The PHP14 expressing cell shown the protrusive edges formation and PHP14, Arp3, F-actin colocalized to the leading edge (indicated by arrows), while PHP14 knockdown cell exhibited diffuse cytoplasmic staining of Arp3. Scale bar 10 μm. (B) LX-2 cells were transfected with PHP14-pEGFP for 48 hours, and treated with CK-666 (150 μM), then imaged for a total time of 20 min at 1 min intervals. Select time points shown the localization of PHP14 gradually disappeared at the leading edge after CK-666 treatment (indicated by arrows). Scale bar 10 μm. (C) His-tagged PHP14 (4 mM, 14 kDa) or bovine serum albumin (BSA, 4 mM, 66 kDa) was added to pre-polymerized actin (2 mM, 42 kDa), incubated for 30 min, and subjected to the F-actin binding assay. The supernatant (S) and pellet fractions (P) were separated by SDS-PAGE. Co-sedimentation with purified non-muscle actin and His-tagged PHP14 revealed that PHP14 binds to F-actin. (D) LX-2 cells were grown to 100% confluence, and stained for PHP14, Arp3 and F-actin, the PHP14 co-localization with F-actin fibers was observed. Scale bar 10 μm.

cytoskeleton is performed by several F-actin-binding proteins, including WAVEs, Arp2/3, IQGAP and cortactin.[15-18] Therefore, we examined whether PHP14 also takes part in the reorganization of the actin cytoskeleton at the leading edge, and thus affecting lamellipodia formation. As we expected, we observed a rapid recruitment of PHP14 to the membrane protrusion sites upon PDGF induction. Moreover, this recruitment occurred in parallel to Arp3. The Arp2/3 complex is an evolutionarily conserved actin nucleation factor localized at the leading edge of motile cells[19-21] that emerges as an important player in the initiation of actin polymerization for actin-based motility.[22,23] Therefore, the concomitant translocation of PHP14 and Arp3 to the leading edge during lamellipodia formation suggests the function of PHP14 in lamellipodia formation, and thus cell motility.

To explore this idea, we investigated the effect of PHP14 knockdown on Arp3 localization and lamellipodia formation. As shown in Fig. 3, the localization of Arp3 at the leading edge was abolished after PHP14 knockdown. Moreover, PHP14 knockdown decreased the localization of Arp3 to the leading edge and lamellipodia formation of HuH-7 cells treated with PDGF. However, the location of PHP14 at the leading edge seemed to depend on Arp2/3, as the localization of PHP14 gradually disappeared, in parallel with Arp3, at the leading edge after the Arp2/3 inhibitor, CK-666, was added (Fig. 4B and S3A). These results demonstrated an association between PHP14 and Arp3 in lamellipodia formation.

However, we did not detect a direct PHP14-Arp3 interaction by a coimmunoprecipitation assay, even when the lamellipodia-inducing factor, PDGF, was present (Fig. S3B). Those results indicated that the interaction between PHP14 and Arp3 in lamellipodia formation might be indirectly. Interestingly, we detected a direct interaction of PHP14 and F-actin in vitro, and a colocalization of PHP14 and F-actin fibers in cells that reached 100% confluence. To our knowledge, this is the first report on the binding of PHP14 to F-actin. PHP14 is a histidine phosphatase, which is similar to the janus proteins of Drosophila; most studies have focused on its phosphorylation function and substrates.[1-6] In previous studies, we have found that PHP14 may be involved in actin cytoskeleton reorganization, because the actin cytoskeleton reorganized after PHP14 knockdown,[7] but the mechanism was nuclear. In this study, we demonstrated that PHP14 may be directly involved in actin cytoskeleton reorganization, as PHP14 was recruited to the leading edge of motile cells, the site of actin remodeling and reorganization. Most importantly, PHP14 affected Arp3 in a functional manner, especially its localization, and thus lamellipodia formation. Purified Arp2/3 complex did not (or poorly) stimulate actin nucleation, but in the presence of nucleation promoting factors, such as Wiskott-Aldrich Syndrome protein family members, the Arp2/3 complex nucleated a new actin filament from the side of an existing filament, thus forming lamellipodia and driving cell motility.[24-27] Based on the above observations, we speculate that the indirect interaction between PHP14 and Arp3 may be mediated by F-actin, and PHP14 may regulate actin remodeling directly, but the precise mechanism should be investigated further.

In conclusion, our study demonstrated that PHP14 regulates cell migration, as we have previously reported, possibly through directly mediating lamellipodia formation.

Materials and methods

Cell culture and reagents

LX-2, HuH-7, BEL7402 and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS, Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin and incubated at 37°C with 5% CO_2. Lipofectamine 3000 transfection reagent was purchased from Invitrogen (USA). PDGF-BB was purchased from PeproTech (Rocky Hill, USA). The Arp2/3 inhibitor CK-666 was purchased from Merk (USA). Primary rabbit polyclonal anti-Arp3 antibody and mouse monoclonal anti-PHP14 antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary

anti-mouse-Alexa 488 antibody, anti-rabbit-Alexa 647 antibody were purchased from Invitrogen (USA). Rhodamine conjugated phalloidin was purchased from Molecular Probe (Eugene, OR). PHP14-pEGFP vector were obtained from GENECHEM (Shanghai, China). BSA protein, and non-muscle actin protein were purchased from Cytoskeleton, Inc. (Denver, CO). His-tagged PHP14 was generated as previous.[7]

RNAi interference

Sense sequences for PHP14 shRNA and control shRNA were as follows: PHP14 RNAi, 5′-CGGACATCTACGA-CAAAGT-3′ and Control RNAi, 5′-TTCTCCGAACGT GTCACGT-3′. All shRNA were synthesized and cloned into a hU6-MCS-SV40-Neomycin vector. The insert-containing vector or control vector was transfected into cells using Lipofectamine 3000 according to the manufacturer's protocol. The culture medium was changed 5 h after transfection. Cells were harvested after 24 h or 48 h for different assays.

Immunofluorescent staining

Cells grown on coverslips were washed 3 times in PBS, fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized for 10 min with 0.3% Triton X-100 in PBS. Nonspecific binding sites were blocked by 1 h incubation in 5% bovine serum albumin in PBS. After 3 5-min wash with PBS, cells were incubated with the primary antibody against PHP14 (1:200) and Arp3 (1:200), 4°C overnight. After 3 5-min wash with PBS, cells were incubated with a mixture of anti-mouse Alex 488 (1:200) and anti-rabbit Alex 647 (1:200) conjugated secondary antibodies for 1 h at room temperature. Following 3 5-min wash with PBS, cells were incubated with rhodamine conjugated phalloidin at 5 U/mL for 30 min. After additional PBS washes, cells were mounted on a slide in mounting medium with DAPI (Molecular Probe, USA). Cells were examined and photographed with a confocal microscope (FV 300, Olympus).

Live cell imaging

Approximately 24 h post-transfection of the LX-2 cells with the PHP14-pEGFP plasmid, cells were seeded into the microscope culture dishes (Corning, USA). Cells were incubated overnight at 37°C prior to initiating the imaging. CK-666 was added and images were acquired every 1 min.

In vitro Actin co-sedimentation Assays

Actin co-sedimentation assays were performed as follows: non-muscle actin derived from human platelets

were purchased (Cytoskeleton Inc., Denver, CO). Actin was mixed in G-buffer (0.5 mM $CaCl_2$, 5 mM Tris-HCl, pH 8.0) to produce an actin stock. Actin was polymerized in actin polymerization buffer (100 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM Tris-HCl, pH 8.0) at room temperature for 1 h and then incubated with His tagged PHP14 or BSA protein for 30 min at room temperature. Actin filaments with bound proteins were pelleted by centrifugation at 100,000$\times$g for 2h at room temperature. Equal amounts of pellet and supernatant were resolved by SDS-PAGE and proteins were visualized by Coomassie blue staining.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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