Migration and retraction of endothelial and epithelial cells require PHI-1, a specific protein-phosphatase-1 inhibitor protein

Nikolaos A. Tountas and David L. Brautigan*

Center for Cell Signaling, University of Virginia School of Medicine, PO Box 800577, Charlottesville, VA 22908, USA

*Author for correspondence (e-mail: db8g@virginia.edu)

Accepted 23 August 2004
Journal of Cell Science 117, 5905-5912 Published by The Company of Biologists 2004
doi:10.1242/jcs.01506

Summary

Cell migration and retraction are interrelated activities that are crucial for a range of physiological processes such as wound healing and vascular permeability. Immunostaining of brain sections for the specific inhibitor of type-1 protein Ser/Thr phosphatase called PHI-1 showed high expression levels in smooth muscle and especially in vascular endothelial cells. During migration of cultured human lung microvascular endothelial cells, endogenous PHI-1 was concentrated to the trailing edge of the cells. Knockdown of PHI-1 using small interfering RNAs reduced by 45% the rate of HeLa cell migration in a wound-healing assay. These cells exhibited an extremely elongated phenotype relative to controls and time-lapse movies revealed a defect in retraction of the trailing edge. Both HeLa and human vascular endothelial cells depleted of PHI-1 showed increased surface areas relative to controls during cell spreading in a replating assay. Analysis of sequential microscopic images demonstrated this was due to a significant decrease in the number of retraction events, whereas protrusive action was unaffected. The Ser/Thr phosphorylation of several signaling, cytoskeletal and focal-adhesion proteins was unchanged in PHI-1-depleted cells, so the target of PHI-1 inhibited protein-phosphatase 1 remains unidentified. Nonetheless, the results show that PHI-1 participates in regulatory events at the trailing edge of migrating cells and modulates retraction of endothelial and epithelial cells.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/117/24/5905/DC1

Key words: Knockdown, siRNA, Vascular permeability, Brain immunohistochemistry, RNA interference

Introduction

Cell adhesion and migration are cellular processes of vital importance to embryogenesis, tissue regeneration and repair, and immune surveillance. Deregulation contributes to cancer metastases, inflammation and other diseases. Migration and adhesion are multistep events that need to be spatially and temporally coordinated (Lauffenburger and Horwitz, 1996). Integrin engagement and growth-factor-receptor activation contribute signals to initiate cell migration and adhesion (Ridley et al., 2003). The role of protein tyrosine (Tyr) kinases in transducing these signals has been well studied, whereas the contribution of protein phosphatases has only recently begun to be appreciated (Larsen et al., 2003). In addition to protein Tyr phosphorylation, serine/threonine (Ser/Thr) phosphorylation of various actin-binding proteins controls their activities and triggers the dynamic reorganization of the actin cytoskeleton (Bailly and Jones, 2003; Bretscher et al., 2002). This reorganization is integral to the morphological changes that accompany cell adhesion and migration.

Protein Ser/Thr phosphatase 1 (PP1) is one of the most highly conserved enzymes known and plays a central role in a range of cellular processes including protein synthesis, RNA splicing, cell-cycle progression and glycogen metabolism (Aggen et al., 2000; Ceulemans and Bollen, 2004). Different regulatory subunits associate with the PP1 catalytic subunit to determine the specificity, activity and cellular localization of PP1 holoenzymes for these various physiological processes (Ceulemans and Bollen, 2004; Cohen, 2002). For example, smooth-muscle contraction and the tension exerted by actin microfilaments in non-muscle cells are regulated by myosin light chain (MLC) phosphorylation (Webb, 2003). The MLC phosphatase (MLCP) is a specialized holoenzyme composed of PP1 catalytic subunit and regulatory subunits that associates with stress fibers and myofilaments (Hartshorne, 1998). Dephosphorylation of MLC by MLCP results in smooth-muscle relaxation. The response is modulated by hormonal stimuli via phosphorylation of the MLCP inhibitor protein CPI-17 (Somlyo and Somlyo, 2003).

There are multiple inhibitor proteins specific for PP1 that do not inhibit the related Ser/Thr phosphatases PP2A and PP2B (Eto et al., 2003), which have their own dedicated inhibitor proteins. Inhibitor-1 and DARPP-32 require phosphorylation by cAMP-dependent protein kinase to be potent inhibitors of PP1. Alternatively CPI-17 is the founding member of a family of PP1-specific inhibitors that are phosphorylated and activated by protein kinase C (PKC), as well as other kinases. PHI-1 is a 17 kDa PP1 inhibitor protein closely related in amino acid sequence to CPI-17. PHI-1 is capable of inhibiting PP1 as well

as different PP1 holoenzymes, hence the name phosphatase holoenzyme inhibitor 1 (Eto et al., 1999). The inhibitory potency of PHI-1 against PP1 is augmented by phosphorylation, but no physiological kinases or signaling pathways other than PKC that are responsible for PHI-1 phosphorylation are yet known.

We recently reported that PHI-1 is present at the highest concentrations in smooth muscles and endothelial cells (Tountas et al., 2004). Based on this information, we sought to define a functional role for PHI-1 in these cells. In the present study, we report that PHI-1 regulates the migration of human endothelial and epithelial cells. We show that PHI-1 localizes to the retracting end of migrating endothelial cells. Silencing of PHI-1 expression using small interfering RNAs (siRNAs) causes a severe decrease in the rate of cell migration and an extremely elongated cell phenotype. Paradoxically, PHI-1 depletion increases the rate of cell spreading. We demonstrate that, during cell spreading, the rate of cell retraction is significantly reduced when PHI-1 is knocked down, whereas the rate of protrusion is unaffected. Finally, we show that PHI-1 does not seem to alter the actin cytoskeleton or the Ser/Thr phosphorylation status of certain F-actin-interacting proteins that are known to be PP1 substrates. Our results imply that PHI-1 and PHI-1-sensitive PP1 holoenzymes regulate cell retraction.

Materials and Methods

Cell cultures

Human lung-microvascular endothelial cells (HMVEC-L) and human umbilical-vein endothelial cells (HUVEC) were grown in EGM-2-MV and EGM-2 media, respectively (Cambrex BioScience Walkersville, Walkersville, MD). HeLa cells were obtained from the University of Virginia Tissue Culture Facility and grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Immunofluorescent microscopy

Adult rat brains were dissected, fixed in room-temperature 4% paraformaldehyde buffered with PBS pH 7.4 for 1 hour, dehydrated, and embedded in paraffin for sectioning. Sections were rehydrated and blocked in 1% goat serum, followed by incubation for 1 hour in rabbit anti-PHI-1 antibody (Tountas et al., 2004) and mouse anti-α-smooth-muscle-actin antibody (Sigma, St Louis, MO). Control sections were incubated with pre-immune rabbit serum or anti-PHI-1 antibody preadsorbed to PHI-1 recombinant protein. For immunodetection of PHI-1, anti-rabbit-IgG-alkaline phosphatase (AP) antibody (Vectastain ABC-AP; Vector Laboratories, Burlingame, CA) and the Vector Red substrate (Vector Laboratories) were used according to the manufacturer's instructions. For immunodetection of α-smooth-muscle actin, anti-mouse-IgG antibody coupled to Oregon Green 488 (Molecular Probes, Eugene, OR) was used. Slides were examined and photographed using an Olympus BX50WI Fluorview confocal microscope.

HMVEC-L were plated on Eppendorf CELLocate microgrid coverslips (Brinkmann, Westbury, NY) that had been coated by incubating overnight with 20 μg ml^{-1} fibronectin (Sigma) in PBS. Cell migration was monitored by time-lapse photomicroscopy, capturing 1 frame per minute for 60 minutes, using a Zeiss Axiovert 135TV inverted microscope with a Hamamatsu Orca II CCD camera and controlled with OpenLab software (Improvision, Lexington, MA). At the end of the 60 minutes, the cells were fixed in 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS, blocked in 10% goat serum for 1 hour and incubated overnight at 4°C in anti-PHI-1 antibody solution in 5% goat serum in PBS. As a control, other specimens were incubated with pre-immune rabbit serum. For immunodetection, cells were incubated in anti-IgG conjugated to Oregon Green 488 (Molecular Probes) to detect PHI-1, and phalloidin/Alexa-Fluor-546 (Molecular Probes) to detect F-actin, and examined and photographed with a Nikon Eclipse E800 microscope equipped with a Hamamatsu Orca CCD camera operated with the OpenLab software. Control HeLa cells or cells with PHI-1 knocked-down by siRNA were plated on fibronectin-coated coverslips and stained for F-actin using phalloidin/Alexa-Fluor-488 as described above.

PHI-1 silencing by siRNA

Two siRNA duplexes (Dharmacon, Lafayette, CO) were used to knock down PHI-1 protein production. The first (siRNA#1) targeted the sequence of PHI-1 that starts at nucleotide (nt) +230 in the coding sequence 5′-AGCAGCTCACGCGCCTCTACG-3′ and the second (siRNA#2) the sequence that start at +363 (5′-AAACCCA-CAGAGGCCTTCATT-3′). BLAST searches of a human expressed sequence tag database produced no matches with other recognized proteins. CPI-17 is the protein most closely related to PHI-1, but it is not expressed in HeLa cells or HUVECs. For the knockdown of α4, a PP2A regulatory subunit, the siRNA duplex targeted the sequence that starts at nt +678: 5′-AAGGCATCAACTTCTAACTCA-3′. Cells were transfected with 120 pmoles of siRNA per 35 mm dish using Oligofectamine (Invitrogen) and following the manufacturer's instructions. Cells were transfected again with fresh reagent 2 days after the first transfection. Cells were lysed on the fourth day in 1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.4 mM Pefabloc (Roche, Indianapolis, IN), and 20 μg total cell protein was analysed by immunoblotting with anti-PHI-1 (Tountas et al., 2004), anti-α4 (Liu et al., 2001) and anti-PP1 (BD Transduction Laboratories, San Diego, CA) antibodies. Antibodies to analyse phosphoproteins in these cells were from Cell Signaling Technology (Beverly, MA) or Biosource (Camarillo, CA).

Cell migration assay

Confluent HeLa-cell monolayers grown in plastic 35 mm dishes were scraped with a razor blade on the third day of the siRNA transfection protocol. The cells were incubated for 17 hours and then photographed, lysed and analysed for PHI-1 knockdown by immunoblotting. The distance migrated by the cells in the 17-hour period was measured using the ruler tool in OpenLab and is reported in pixels (0.7 pixels per μm at 100× magnification).

Cell spreading assay

HeLa cells or HUVECs that were on day 4 of the siRNA transfection protocol were trypsinized and replated on plastic 35 mm dishes. Replated cells were incubated for 15 minutes and spreading was monitored by time-lapse photomicroscopy as described above.

Results

PHI-1 localizes to the endothelia of large and small blood vessels in brain

We stained rat brain sections for PHI-1 with an affinity-purified antibody and for smooth-muscle actin with a monoclonal antibody (Fig. 1). The brain tissue was negative for PHI-1 and smooth-muscle actin (dark background). The muscular layer of blood vessels stained intensely for smooth-muscle actin (Fig. 1A) and PHI-1 was present in both the muscular and

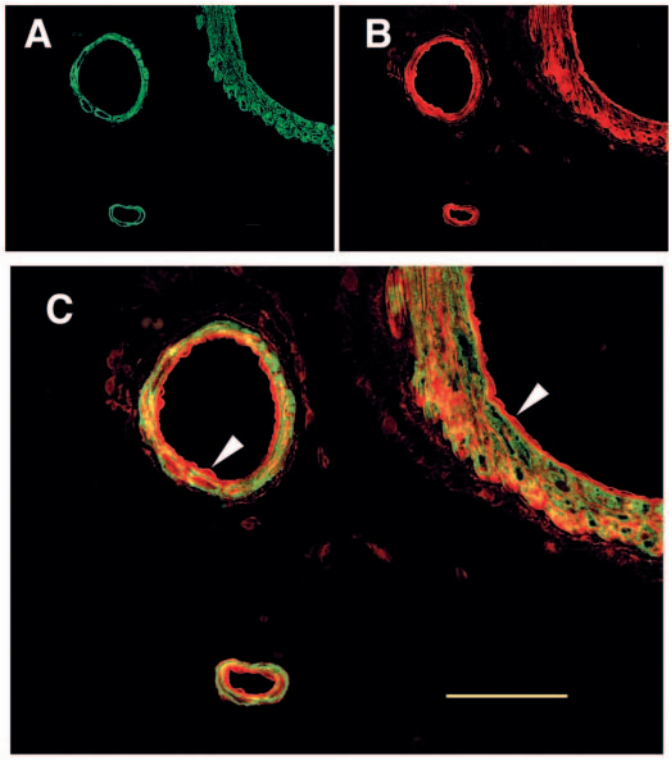

Fig. 1. Vascular smooth-muscle and endothelial localization of PHI-1 in rat brain sections. (A) α-Smooth-muscle-actin immunostaining (green). (B) PHI-1 immunostaining (red). (C) Overlay of A and B. Original magnification was 600×; bar in C, 50 μm.

endothelial regions (Fig. 1B), the latter clearly evident as the red-stained layers lining the vessel walls in the overlaid images in Fig. 1C (white arrows). These results are consistent with our recent report using human and mouse tissue microarrays, showing that PHI-1 localizes preferentially to visceral and vascular smooth muscles and endothelial cells (Tountas et al., 2004).

PHI-1 concentrates at the trailing edge of migrating endothelial cells

To investigate the function of PHI-1, we studied its intracellular localization in HMVEC-L cells. We seeded HMVEC-L cells onto fibronectin-coated glass coverslips and followed migration by time-lapse photomicroscopy. HMVEC-L cells randomly migrated ~20 μm over 60 minutes. Cells were fixed and stained for PHI-1 (green) and for F-actin (red) (Fig. 2). An unexpected observation was the pronounced concentration of PHI-1 at the trailing edge of migrating cells (Fig. 2A,B). Migrating cells were identified by the lamellipodia spreading at the front edge and the presence of actin-rich retraction fibers (stained red) radiating from the rear edge of the cells. Cells in Fig. 2A-C are depicted as migrating from left to right. The nuclei were stained green in all the cells but this was also seen in the control (Fig. 2C) that was incubated with pre-immune rabbit serum instead of primary anti-PHI-1 antibody. Thus, the nuclear staining was nonspecific. Comparison of Fig. 2C with Fig. 2A,B reveals the specific anti-PHI-1 localization at the trailing edge. In contrast to migrating HMVEC-L cells, PHI-1

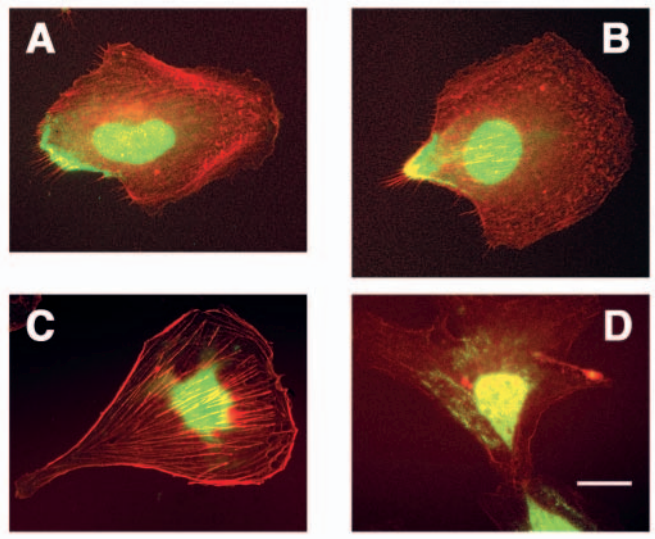

Fig. 2. Localization of endogenous PHI-1 to the trailing edge of HMVEC-L cells. (A,B) Migrating HMVEC-L cells stained for PHI-1 (green) and F-actin (red). (C) Migrating cell stained with pre-immune rabbit serum instead of anti-PHI-1 antibody, as a control. (D) Non-migrating cell stained for PHI-1 and F-actin. Magnification was 600×; bar in D, 15 μm.

was not concentrated to the trailing edge in non-migrating cells but displayed a more diffuse cytoplasmic distribution (Fig. 2D). The localization of endogenous PHI-1 to the trailing edge of migrating HMVEC-L cells suggested to us that PHI-1 might be involved in cell migration.

Knockdown of PHI-1 by siRNA retards HeLa-cell migration in a wound-healing assay

To investigate the function of endogenous PHI-1, we ablated the production of PHI-1 using RNA interference. Synthetic siRNA duplexes matching different regions of the PHI-1 sequence were tested for their ability to knock down PHI-1 protein expression. A series of preliminary experiments showed that optimal reduction in PHI-1 protein levels was achieved after 4 days, especially when sequential transfections were done at days 0 and 2. The siRNA duplexes were effective at knocking down PHI-1 production in HeLa cells, with slightly different efficiencies: siRNA#1 produced a ~80-90% reduction, whereas the knockdown was virtually 100% for siRNA#2 (Fig. 3A). PHI-1 knockdown did not alter the expression of an unrelated PP2A regulatory protein, α4, used as a control and, conversely, the knockdown of α4 protein did not alter the expression of PHI-1 (Fig. 3A). Additional controls demonstrated the specificity of the siRNA knockdown of PHI-1. Levels of PP1 (Fig. 3A) (the physiological target of PHI-1) and inhibitor-2 (not shown) (another PP1 inhibitory protein) were unchanged.

We tested the effect of knocking down PHI-1 on cell migration in an in vitro wound-healing assay using either siRNA#1 or siRNA#2. Cells were sequentially transfected on days 0 and 2 with siRNA and grown for a total of 3 days into a confluent monolayer. The HeLa-cell monolayer was ‘wounded’ with a razor blade by scraping a path clear of cells. Cells migrated from the edge of the remaining monolayer into

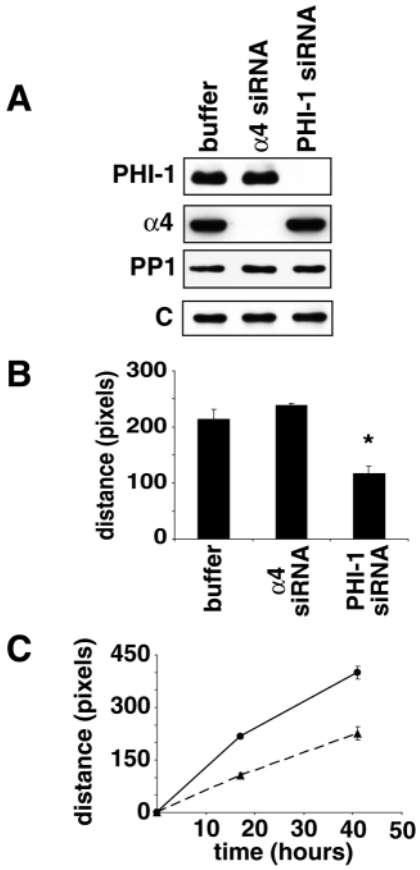

Fig. 3. PHI-1 depletion by siRNA retards HeLa cell migration. (A) Total cell lysates (20 μg per lane) from cells transfected with buffer, α4 siRNA or PHI-1 siRNA#2 resolved by SDS-PAGE and blotted for PHI-1, α4 and PP1. 'C' is a band present in PHI-1 immunoblots that serves as loading control. (B) Quantitation of the distance of migration 17 hours after scraping of cells transfected with buffer, α4 siRNA or PHI-1 siRNA#2 (*P<0.05). (C) Kinetics of cell migration under PHI-1 depletion. Same as B, except that cell migration was followed for a total of 41 hours after scraping (circles represent control cells and triangles represent PHI-1-knocked-down cells).

the open area ('wound'). The distance from the leading edge of the migrating cells to the mark of the initial wound was measured 17 hours later. Cells depleted of PHI-1 using siRNA#2 exhibited a 45% decrease (Fig. 3B) in the distance migrated compared with mock-transfected cells that continued to express PHI-1. A significant decrease (P<0.05) in the distance of migration after knockdown of PHI-1 was independently reproduced with siRNA#1, strengthening the conclusion that the decreased cell migration was a specific effect of PHI-1 depletion. However, knockdown of the PP2A regulatory protein α4, the mammalian homolog of yeast Tap42, had no significant effect on cell migration in this assay (Fig. 3B).

We examined the kinetics of cell migration with cells depleted of PHI-1 by measuring the distance traveled at different times in the wound-healing assay. The directional migration of cells adjacent to the wound was monitored by microscopy as they repopulated the exposed area. Cells transfected with siRNA for PHI-1 migrated at a 45% lower speed (5.5 pixels per hour rather than 9.7 pixels per hour) than mock-transfected cells (Fig. 3C). At the end of the migration period (41 hours), the cells were lysed and immunoblotted for PHI-1, confirming that PHI-1 protein was essentially absent from the siRNA-treated cells during the entire duration of the experiment (not shown). We concluded that depletion of PHI-1 by siRNA retarded the rate of cell migration.

One might predict that elevated levels of PHI-1 accelerate cell migration, because reduced levels of PHI-1 retarded cell migration. We attempted to overexpress epitope-tagged PHI-1 by transient transfection but, within hours, cells detached from the dishes. Modifying the conditions (e.g. less plasmid DNA, various times of transfection, precoating surfaces etc.) did not alter the outcome of cell detachment. The overexpression of PHI-1 seemed to cause loss of cell adhesion, which could be considered the opposite of retarded migration owing to defective rear release in cells depleted of PHI-1 (see below). Regardless, we were unable to obtain cells with substantially elevated levels of PHI-1 to assay for changes in migration or adhesion. These results were consistent with the cell detachment and hypercontraction produced in fibroblasts by overexpression of the closely related PP1 inhibitor protein CPI-17 (Eto et al., 2000).

Cell retraction during migration is blocked by siRNA depletion of PHI-1

To examine the involvement of PHI-1 in the multistep, dynamic process of cell migration we produced time-lapse movies of control and siRNA-transfected HeLa cells. Cells were replated at low density and their migratory behavior followed for 5 hours by acquiring phase-contrast images at 5-minute intervals. Control cells expressing endogenous PHI-1 (Fig. 4A) exhibited extension at both ends of the cell with dynamic lamellipodial ruffling for a period of over 1 hour, followed by rapid retraction of one end of the cell within a 5-10-minute period (Fig. 4A, white arrows). Cells typically extended to ~60 μm in length before retracting. On average, 80% of the cells in each experiment exhibited cycles of extension and retraction with two or three retraction events per cell over a 5-hour period. By contrast, cells with siRNA knockdown of PHI-1 also showed lamellipodial activity at both ends and bipolar extension, the same as control cells, but did not undergo retraction and therefore produced an extremely elongated phenotype (Fig. 4B). Cells reached lengths of ~150 μm in the 5-hour period. On average, 90% of the cells adopted this elongated phenotype. We concluded that PHI-1 was required for the release and retraction of HeLa cells during cell migration.

Cell spreading after replating is accelerated by knockdown of PHI-1

Cells undergo rounds of protrusion and retraction during the process of spreading after replating. We examined the role of PHI-1 in spreading of HeLa cells or HUVECs. Cells were transfected with PHI-1 siRNA for 4 days or mock transfected as controls. The cells were trypsinized and replated for 30 minutes, and then the cell surface areas were measured by image processing and compared. Knockdown of PHI-1 resulted in a 23% increase in average surface area of HeLa cells (P<0.05;

not shown) and a 13% increase of average area of HUVECs (P<0.05; Fig. 5A). To examine the kinetics of cell spreading, we determined the surface areas of cells at 4 minute intervals. As shown in Fig. 5B, control cells showed a slow rate of spreading over 30 minutes (4.7 square pixels per minute). Cells depleted of PHI-1 by siRNA showed about a 4.5-fold increase in rate of spreading (22.2 square pixels/minute). The velocity of cell spreading (square pixels per minute) in cells with or without PHI-1 was significantly different (P<0.05; Fig. 5C).

Cell retraction but not cell protrusion depends on PHI-1

We identified and scored individual cell-membrane protrusion and retraction events to assess the effects of PHI-1 knockdown. The series of phase-contrast images collected as time-lapse movies during cell spreading assays were converted frame by frame into binary layer images and each one was overlayed onto the previous frame. In this way, the differences in cell area and perimeter were exposed at 30-second intervals (Fig. 6A). Added areas were identified as protrusion events and reduced areas were scored as cell retractions. The number of protrusion events was not significantly different between control HUVECs and HUVECs with PHI-1 knocked down by siRNA (Fig. 6B, black bars). However, cells with PHI-1 knocked down exhibited substantially fewer retraction events than control cells (29±0.33 vs 44±4.6, respectively) (P<0.05; Fig. 6B, gray bars). These results demonstrated that reducing the levels of PHI-1 did not affect the protrusion of the cell edges but did inhibit cell retraction.

Phosphorylation of Ser/Thr in various cytoskeleton and focal-adhesion proteins

We imagined that the defect in retraction by cells depleted of PHI-1 could have been caused by reduced contractile force exerted by actomyosin stress fibers. To test this hypothesis, we stained for F-actin in control HUVECs and HeLa cells (Fig. 7A), and compared these with cells that had PHI-1 knocked down by siRNA (Fig. 7B). There was no noticeable difference between the distributions of microfilaments, making it unlikely that the effects of PHI-1 depletion were mediated by gross alteration of F-actin structures. We also examined the Ser/Thr phosphorylation status of several proteins that are substrates of PP1 and are involved in cell contraction and F-actin organization. The rationale was that PHI-1 depletion would relieve the inhibition of specific PP1 holoenzymes, leading to more active PP1 and relative dephosphorylation of one or more specific PP1 substrates. Phosphorylation of the ezrin/radixin/moesin (ERM) group of actin-binding proteins and MLC regulates their interactions with actin and these proteins are dephosphorylated by PP1. Neither ERM nor MLC phosphorylation status was affected by PHI-1 knockdown, based on blotting using phosphorlyation-site-specific antibodies (Fig. 7C). Cofilin is another PP1 substrate that participates in the cycle of actin polymerization and depolymerization. Immunoblotting in multiple experiments showed minor variations but no significant difference in the phosphorylation of cofilin in control cells compared with cells with PHI-1 knocked down (see representative immunoblot in Fig. 7C). These results were consistent with the unchanged

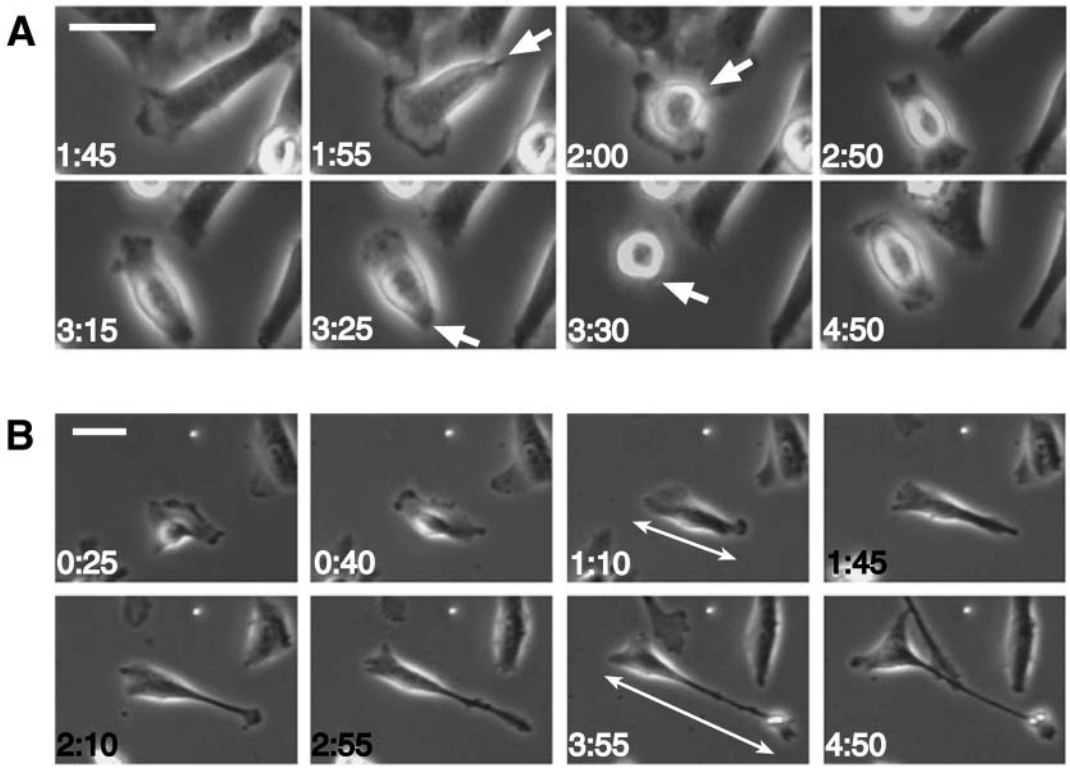

Fig. 4. PHI-1 depletion using siRNAs results in extremely elongated HeLa cells that fail to retract. (A) Time-lapse phase-contrast photomicroscopy of control cell exhibiting two cycles of protrusions and retractions (white arrows) in a 3-hour period. Original magnification was 160×; bar, 35 μm. (B) Time-lapse phase-contrast photomicroscopy of a cell depleted of PHI-1 extending along the direction of the double-headed arrow with no retraction events in 5-hour period. Original magnification was 100×; bar, 40 μm. See Movie 1 and 2 in supplementary material.

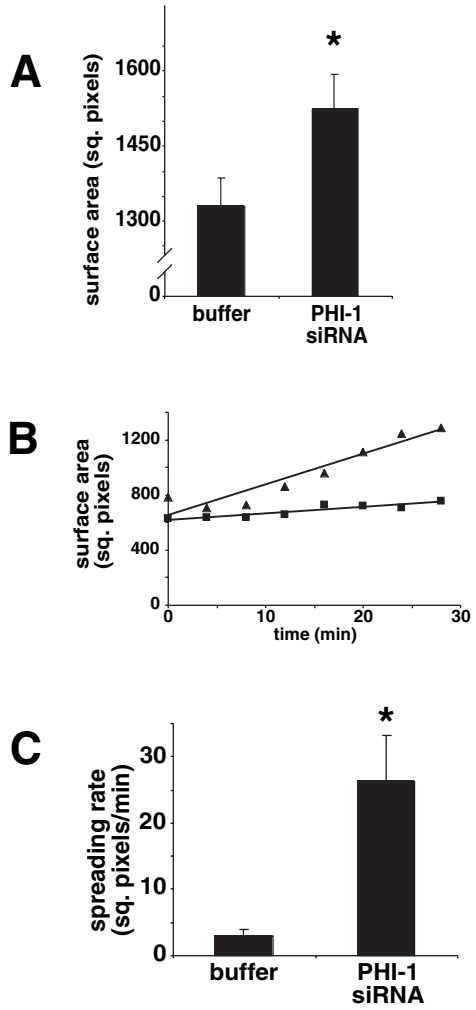

Fig. 5. PHI-1 depletion by siRNAs accelerates HUVEC spreading. (A) HUVEC knocked down for PHI-1 exhibited a 13% larger surface area after 30 minutes than control cells (**P*<0.05, *n*=30). (B) Kinetics of cell spreading for a control (squares) vs a PHI-1 knockdown HUVEC. The slopes of the lines represent the rate of cell spreading. (C) Rate of cell spreading of control vs PHI-1 knocked-down HUVECs (**P*<0.05, *n*=3).

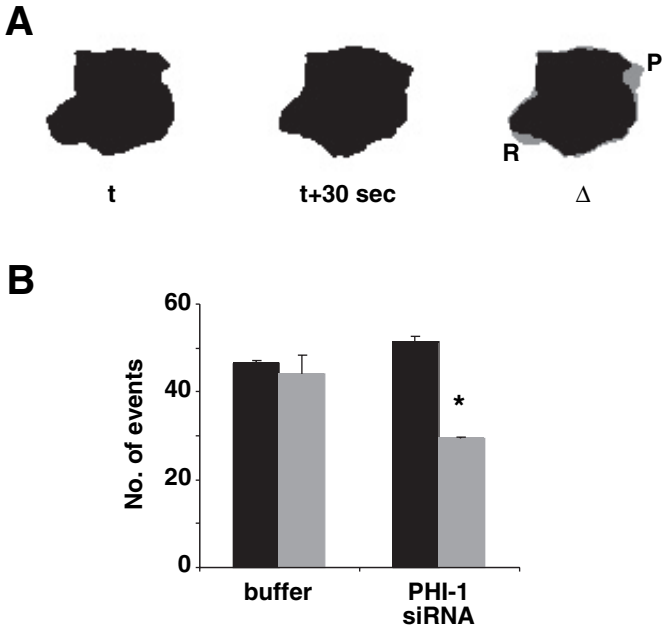

Fig. 6. PHI-1 knockdown by siRNA inhibits lamellipodium retraction but not protrusion in HUVECs. (A) The surface areas of replated cells were compared by overlaying binary images of cells acquired every 30 seconds. The absolute difference between the two surfaces (Δ) was used to determine protrusion (P) and retraction (R) events. (B) The number of protrusion events (black bars) between control and PHI-1 knockdown HUVECs was not significantly different. However, the number of retraction events in PHI-1-knockdown cells was significantly reduced compared with control cells (29±0.3 vs 44±4.4, respectively; **P*<0.05, *n*=3).

appearance of F-actin stress fibers in cells with PHI-1 levels knocked down.

An alternative hypothesis for the defect in cell retraction is impaired release of focal adhesions from the extracellular matrix. Many focal-adhesion proteins are phosphorylated on Ser/Thr residues and, although the functional effects of these phosphorylations are not well understood, they might be related to adhesion turnover (Webb et al., 2004). We examined the phosphorylation of focal-adhesion kinase (FAK) at Ser722 (Fig. 7C) and Ser910 (not shown) with phosphorylation-site-specific antibodies, but neither site was grossly affected by knockdown of PHI-1. These sites in FAK do indeed undergo dynamic, large changes in phosphorylation and we have seen greater than fivefold changes in FAK-Ser722 phosphorylation by immunoblotting in response to replating on fibronectin after suspension (our unpublished observations). The Ser phosphorylation of paxillin, another focal-adhesion protein, was assessed by reduced mobility during SDS polyacrylamide-gel electrophoresis (SDS-PAGE), which has been used as an indirect assay (Ito et al., 2000; Terfera et al., 2002; Young et al., 2003), and no difference was evident between control and PHI-1 knockdown (Fig. 7C). Lastly, extracellular-signal-regulated kinase (ERK), a mitogen-activated-protein kinase, has been recognized as a kinase in focal adhesions and is thought to be responsible for the phosphorylation of multiple substrates. Phosphorylation-site-specific antibodies against the dual Tyr and Thr phosphorylation sites responsible for activation did not detect a difference in ERK1/2 between control and PHI-1 knockdown cells (Fig. 7C). In multiple independent experiments, no significant changes in Ser/Thr phosphorylation were found for these various actin-associated or focal-adhesion proteins.

Discussion

In this report, we provide evidence that the PP1-specific inhibitor protein PHI-1 is required for retraction during migration and spreading of endothelial and epithelial cells. We found that PHI-1 was expressed selectively in the smooth-muscle and endothelial layers of blood vessels in rat brain but not in the surrounding tissue. Endothelial cells from different sizes of blood vessels display different gene-expression profiles (Chi et al., 2003), which might be related to differences in mechanical and structural characteristics and responses to hemodynamics. In this respect, it is interesting that PHI-1 was present in both wide and narrow blood vessels, suggesting that

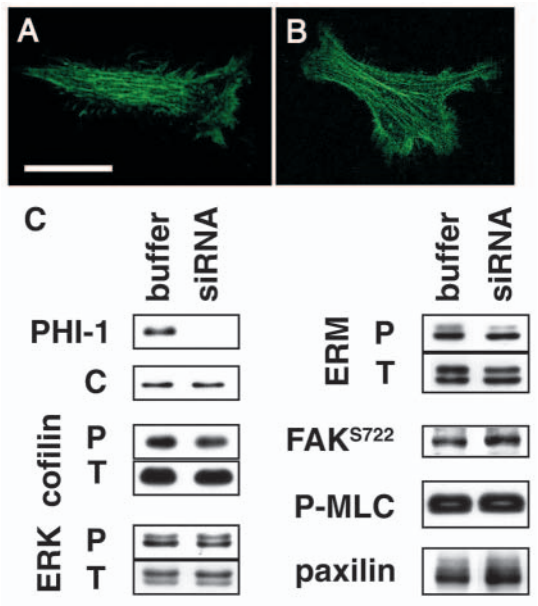

Fig. 7. PHI-1 knockdown by siRNA does not affect stress fibers or the phosphorylation state of selected cytoskeleton and focal-adhesion proteins. (A) Control HeLa cells stained for actin stress fibers. (B) PHI-1-depleted HeLa cells stained for actin stress fibers. Magnifications for A and B were 600×; bar for A and B, 25 μm. (C) Immunoblots of total cell lysates (20 μg per lane) from control and PHI-1-depleted HeLa cells were resolved by SDS-PAGE and blotted for PHI-1, phosphorylated and total cofilin, phosphorylated and total ERK, phosphorylated (P) and total (T) ERM, FAK phosphorylated on Ser722, phosphorylated MLC, and paxilin. 'C' is a band present in PHI-1 immunoblots that serves as loading control.

it has some basic functional role in endothelial cells. The tissue distribution prompted us to study the function of PHI-1 specifically in cultured endothelial cells. Surprisingly, PHI-1 was highly concentrated along the trailing edge of migrating HMVEC-L endothelial cells. This localization was unexpected for a small (17 kDa) soluble protein such as PHI-1. Indeed, PHI-1 displayed diffuse cytoplasmic staining in non-migrating HMVEC-L endothelial cells. These findings suggested to us that, during cell migration, specialized protein complexes are assembled at the trailing edge and PHI-1 is recruited into these complexes. Presumably, PHI-1 is recruited to the trailing edge by binding to a complex that includes PP1, which itself binds PHI-1 with high affinity. It is also possible that PHI-1 binds to other, unidentified proteins. Recent reports with the PP1 inhibitor proteins I-1 and I-2 have shown they bind other targets in addition to PP1 itself (Connor et al., 2001; Eto et al., 2002; Terry-Lorenzo et al., 2002; Wang and Brautigan, 2002). Based on the phenotypes produced by PHI-1 knockdown, these proposed complexes are expected to have an important role in control of cell retraction and cell migration.

Knocking down PHI-1 protein expression using siRNAs had pronounced and specific effects on cell migration. Different sequences were used for synthesis of two siRNA molecules that yielded essentially identical phenotypes. This supported the idea that the phenotypes were caused by depletion of PHI-1, not another protein or other proteins in addition to PHI-1. As further controls for specificity, we demonstrated the levels of PP1 (the presumed target of PHI-1) or another PP1 inhibitor protein, or an unrelated PP2A subunit protein were all unchanged by the siRNA coding for PHI-1. Single transfection of HeLa cells with the different siRNA for PHI-1 produced only a partial knockdown of PHI-1, whereas, in our hands, a sequential transfection protocol produced a virtually complete knockdown of PHI-1 protein (this was also the case with specific siRNAs for other proteins). Apparently, the second transfection boosts the effects and/or ensures that most cells are transfected. Knockdown of PHI-1 decreased the rate of directional cell migration relative to controls in a wound-healing assay. Monitoring random cell migration using time-lapse microscopy movies revealed that control HeLa cells exhibited ameboid movement with a cycle of elongation and retraction every 1.5-2.0 hours (Fig. 4, see Movie 1 in supplementary material). By contrast, cells depleted of PHI-1 exhibited an exaggerated elongated phenotype because the trailing edge of the cells failed to release even after 5 hours (Fig. 4, see Movie 2 in supplementary material), suggesting a defect in the release and retraction of the trailing edge.

Furthermore, we examined how PHI-1 knockdown influenced the rate of cell spreading in a replating assay and found that both HeLa and HUVEC cells depleted of PHI-1 spread significantly more quickly than the corresponding controls, covering a larger surface area over time. At first thought, faster cell spreading seems to be paradoxical given the slower cell migration. More detailed analysis of the protrusion and retraction events of lamellipodia during the spreading process determined that there were significantly fewer retraction events in cells depleted of PHI-1 than controls, whereas the protrusive activity was not different between the two cell populations. Because cells depleted of PHI-1 did not efficiently retract their lamellipodia during spreading, most protrusive events were, in effect, irreversible. However, with control cells, extension and retraction were nearly balanced, with net spreading caused by there being slightly more extension than retraction. Thus, it was a defect in cell retraction caused by reduced levels of PHI-1 that both retarded cell migration and accelerated cell spreading.

How does depletion of PHI-1 cause a defect in cell retraction? One possibility is that PHI-1, like the closely related protein CPI-17, regulates actomyosin contractility by inhibiting myosin phosphatase, a specialized form of PP1. If so, knockdown of PHI-1 would relieve inhibition of the phosphatase and result in decreased phosphorylation of MLC. However, there was no difference in the level of MLC (Ser19) phosphorylation between control and PHI-1-depleted HeLa cells and HUVECs. Also, immunofluorescence of the actin cytoskeleton revealed no differences in stress fiber organization when PHI-1 was knocked down. Furthermore, the levels of site-specific Ser/Thr phosphorylation of cofilin (which controls actin-fiber stability) and ERM proteins (which cross-link actin filaments to the plasma membrane) were unaffected by knockdown of PHI-1. These results are consistent with the unchanged protrusive activity of cells depleted of PHI-1.

An alternative is that PHI-1 might act to promote the release of cells from the substratum and that its knockdown therefore inhibits release of the trailing edge of migrating cells or protrusive edges of spreading cells. The release process itself is not well understood and operates in the context of extensive cytostructural reorganization. Recent work suggests that phosphorylation of Ser/Thr residues in focal-adhesion proteins

such as FAK and paxillin promotes the dynamic turnover of adhesions (Webb et al., 2004). Therefore, depletion of PHI-1 would be predicted to diminish focal-adhesion turnover if these sites in FAK and paxillin were substrates of a PHI-1-inhibited form of PP1. However, we detected no changes in phosphorylation of Ser722 or Ser910 in FAK, or in Ser sites in paxillin that account for reduced mobility by SDS-PAGE. Proteolysis of focal-adhesion proteins by calpain is another process linked to cell release (Huttenlocher et al., 1997) and ERK phosphorylation is reported to activate calpain (Glading et al., 2004). However, phosphorylation of the dual phosphorylation activation site in ERK1/2 was unchanged with knockdown of PHI-1. This agreed with no change in Ser910 phosphorylation of FAK, which is reportedly phosphorylated by ERK2 (Hunger-Glaser et al., 2004). Calpain phosphorylation by ERK2 was unlikely to increase in response to knockdown of PHI-1 because ERK2 did not change. Knockdown of PHI-1 would be predicted to increase, not decrease, phosphatase activity and thereby lower levels of phosphorylation. PHI-1 might reduce the stability of focal adhesions by inhibiting a PP1 complex and thereby increasing Ser/Thr phosphorylation of proteins. Finding this complex and these phosphoprotein substrates is a challenge for future efforts.

Finally, the integrity of the endothelium is crucial for vascular homeostasis. Migration of lymphocytes through the endothelium (diapedesis) is required to combat infections and secreted histamine and thrombin have a role in regulating the barrier function of the endothelial cell layer. Pathological compromise of the barrier function results in increased permeability and thrombotic reactions. Endothelial cell retraction is one mechanism for increased permeability. Previous work has implicated PP1 as a key regulator of the process (Verin et al., 2000; Verin et al., 1995). Our evidence for PHI-1 involvement in endothelial cell retraction is consistent with this concept. The possibility that PHI-1 and PP1 are involved in the regulation of the barrier function of the endothelium poses new opportunities for pharmacological interventions.

We thank A. D. Everett for assistance with immunohistochemistry and A. F. Horwitz and D. J. Webb for helpful discussions. This research was supported by grants (to D.L.B.) CA40042 from the National Cancer Institute and GM56362 from the National Institute of General Medical Sciences, US Public Health Service.

References

Aggen, J. B., Nairn, A. C. and Chamberlin, R. (2000). Regulation of protein phosphatase-1. *Chem. Biol.* **7**, R13-R23.

Bailly, M. and Jones, G. E. (2003). Polarised migration: cofilin holds the front. *Curr. Biol.* **13**, R128-R130.

Bretscher, A., Edwards, K. and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat. Rev. Mol. Cell Biol.* **3**, 586-599.

Ceulemans, H. and Bollen, M. (2004). Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* **84**, 1-39.

Chi, J. T., Chang, H. Y., Haraldsen, G., Jahnsen, F. L., Troyanskaya, O. G., Chang, D. S., Wang, Z., Rockson, S. G., van de Rijn, M., Botstein, D. et al. (2003). Endothelial cell diversity revealed by global expression profiling. *Proc. Natl. Acad. Sci. USA* **100**, 10623-10628.

Cohen, P. T. (2002). Protein phosphatase 1 – targeted in many directions. *J. Cell Sci.* **115**, 241-256.

Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M. and Shenolikar, S. (2001). Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol. Cell. Biol.* **21**, 6841-6850.

Eto, M., Karginov, A. and Brautigan, D. L. (1999). A novel phosphoprotein inhibitor of protein type-1 phosphatase holoenzymes. *Biochemistry* **38**, 16952-16957.

Eto, M., Wong, L., Yazawa, M. and Brautigan, D. L. (2000). Inhibition of myosin/moesin phosphatase by expression of the phosphoinhibitor protein CPI-17 alters microfilament organization and retards cell spreading. *Cell Motil. Cytoskeleton* **46**, 222-234.

Eto, M., Elliott, E., Prickett, T. D. and Brautigan, D. L. (2002). Inhibitor-2 regulates protein phosphatase-1 complexed with NimA-related kinase to induce centrosome separation. *J. Biol. Chem.* **277**, 44013-44020.

Eto, M., Leach, C., Tountas, N. A. and Brautigan, D. L. (2003). Phosphoprotein inhibitors of protein phosphatase-1. *Methods Enzymol.* **366**, 243-260.

Glading, A., Bodnar, R. J., Reynolds, I. J., Shiraha, H., Satish, L., Potter, D. A., Blair, H. C. and Wells, A. (2004). Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol. Cell. Biol.* **24**, 2499-2512.

Hartshorne, D. J. (1998). Myosin phosphatase: subunits and interactions. *Acta Physiol. Scand.* **164**, 483-493.

Hunger-Glaser, I., Fan, R. S., Perez-Salazar, E. and Rozengurt, E. (2004). PDGF and FGF induce focal adhesion kinase (FAK) phosphorylation at Ser-910: dissociation from Tyr-397 phosphorylation and requirement for ERK activation. *J. Cell. Physiol.* **200**, 213-222.

Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A., Ginsberg, M. H. and Horwitz, A. F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* **272**, 32719-32722.

Ito, A., Kataoka, T. R., Watanabe, M., Nishiyama, K., Mazaki, Y., Sabe, H., Kitamura, Y. and Nojima, H. (2000). A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. *EMBO J.* **19**, 562-571.

Larsen, M., Tremblay, M. L. and Yamada, K. M. (2003). Phosphatases in cell-matrix adhesion and migration. *Nat. Rev. Mol. Cell Biol.* **4**, 700-711.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Liu, J., Prickett, T. D., Elliott, E., Meroni, G. and Brautigan, D. L. (2001). Phosphorylation and microtubule association of the Opitz syndrome protein mid-1 is regulated by protein phosphatase 2A via binding to the regulatory subunit alpha 4. *Proc. Natl. Acad. Sci. USA* **98**, 6650-6655.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.

Somlyo, A. P. and Somlyo, A. V. (2003). Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83**, 1325-1358.

Terfera, D. R., Brown, M. C. and Turner, C. E. (2002). Epidermal growth factor stimulates serine/threonine phosphorylation of the focal adhesion protein paxillin in a MEK-dependent manner in normal rat kidney cells. *J. Cell. Physiol.* **191**, 82-94.

Terry-Lorenzo, R. T., Elliot, E., Weiser, D. C., Prickett, T. D., Brautigan, D. L. and Shenolikar, S. (2002). Neurabins recruit protein phosphatase-1 and inhibitor-2 to the actin cytoskeleton. *J. Biol. Chem.* **277**, 46535-46543.

Tountas, N. A., Mandell, J. W., Everett, A. D. and Brautigan, D. L. (2004). Juxtamembrane localization of the protein phosphatase-1 inhibitor protein PHI-1 in smooth muscle cells. *Histochem. Cell. Biol.* **121**, 343-350.

Verin, A. D., Patterson, C. E., Day, M. A. and Garcia, J. G. (1995). Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. *Am. J. Physiol.* **269**, L99-L108.

Verin, A. D., Csortos, C., Durbin, S. D., Aydanyan, A., Wang, P., Patterson, C. E. and Garcia, J. G. (2000). Characterization of the protein phosphatase 1 catalytic subunit in endothelium: involvement in contractile responses. *J. Cell. Biochem.* **79**, 113-125.

Wang, H. and Brautigan, D. L. (2002). A novel transmembrane Ser/Thr kinase complexes with protein phosphatase-1 and inhibitor-2. *J. Biol. Chem.* **277**, 49605-49612.

Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T. and Horwitz, A. F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* **6**, 154-161.

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Adv. Physiol. Educ.* **27**, 201-206.

Young, M. R., Liu, S. W. and Meisinger, J. (2003). Protein phosphatase-2A restricts migration of Lewis lung carcinoma cells by modulating the phosphorylation of focal adhesion proteins. *Int. J. Cancer* **103**, 38-44.