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A likely role for the PH-domain containing protein, PEPP2/ PLEKHA5, at the membrane-microtubule cytoskeleton interface

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ABSTRACT: PH (pleckstrin homology) domains are well known to bind membrane phosphoinositides with different specificities and direct PH domain-containing proteins to discrete subcellular compartments with assistances of alternative binding partners. PH domain-containing proteins have been found to be involved in a wide range of cellular events, including signalling, cytoskeleton rearrangement and vesicular trafficking. Here we showed that a novel PH domain-containing protein, PEPP2 (also known as PLEKHA5), displays moderate phosphoinositide binding specificity. Full length PEPP2 was observed to variably associate with both the plasma membrane and microtubules. The membrane-associated PEPP2 nucleated at cell-cell contacts and the leading edge of migrating cells. Overexpression of PEPP2 increased membrane microviscosity, indicating a potential role for PEPP2 in regulating function of microtubule-dependent membrane functions.

Introduction

Phosphatidylinositols are intrinsic components of membrane lipids on the cytoplasmic surface of membranes. The different combination of phosphorylation on the inositol rings gives rise to different phosphoinositides, with their synthesis temporally and spatially controlled by kinases and phosphatases (van den Bout & Divecha, 2009). Phosphoinositides (PI) function through their cytoplasmic effector proteins that contain PI-binding domains by recruiting them onto the location where membrane events take place or assist protein-membrane association via electrostatic interactions. The spatial distribution of phosphoinositides can therefore determine their involvement in different aspects of membrane events (van den Bout & Divecha, 2009).

The first PI-binding domain described was in pleckstrin, a major protein kinase C substrate of platelets (Tyers *et al.*, 1988). Domains showing sequence homology to this region of plekstrin are now known as PH (plekstrin homology) domains. The PH domain is the 11th most common domain and is shared by various proteins, including signaling proteins, trafficking proteins and cytoskeletal proteins and consists of a conserved region of 100-120 amino acids (Lemmon *et al.*, 2007). PH domains have been found in proteins that have binding specificity for all types of phosphoinositides and, as such, are involved in many different cellular processes. For example, the activity of the Bbl family of guanine nucleotide exchange factors (GEFs), which couple receptor signaling with actin organization, are regulated by their interaction with PI(4,5)P_2 through their PH domains

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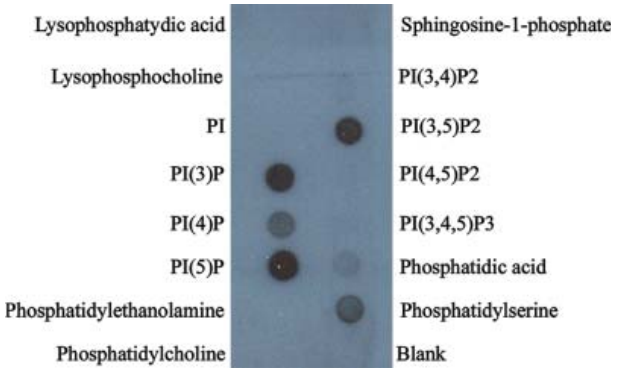

FIGURE 1. Lipid-binding specificity of the PH domain of PEPP2. About 0.5 µg/ml GST and GST-PEPP2PH were used in a protein-lipid overlay assay. The interaction between GST-PEPP2PH and immobilized lipids on the PIP strip was detected using a monoclonal anti-GST antibody. Strong interactions were observed between GST-PEPP2PH and PI(3,5)P2, PI3P, PI4P and PI5P, respectively. No specific binding was seen with GST only (not shown).

(Russo *et al.*, 2001). PEPP2 (also known as PLEKHA5) is a novel PH-domain-containing protein with uncharacterized function. PEPP2 is expressed ubiquitously while two related proteins, PEPP1 (PLEKHA4) and PEPP3 (PLEKHA6), displayed very restricted and low level expression (Dowler *et al.*, 2002). The three proteins share high homology in their PH domains, especially in the putative phosphotidylinositol-binding motif (PPBM) and were proposed to bind phosphotidylinositol-3-phosphate (PI3P).

In this study, we determined the lipid-binding specificity of PEPP2 and its subcellular distribution. Our results showed that unlike PEPP1, the PH domain of PEPP2 actually exhibited less specificity in lipid binding and displayed moderate to strong interactions with PI(5)P, PI (3)P and PI(3,5) P2 *in vitro*. However, the PEPP2-PH domain was unlikely to bind PI-3-phosphate derivatives *in vivo* due to the unaffected membrane-association when treated with a PI3-kinase inhibitor, wortmannin. Full length PEPP2 displayed both a variable plasma membrane-associated and microtubule-associated distribution, suggesting a potential role for PEPP2 in directing events between these two cellular compartments.

Materials and Methods

Miscellaneous enzymes, chemicals and constructs

Restriction endonucleases were purchased from New England Biolabs (Genesearch Pty Ltd, Arundel, Queensland), Klenow fragment from GeneWorks Pty Ltd (Thebarton, South Australia), and both T4 ligase and T4 DNA polymerase from Roche Diagnostics Australia (Castle Hill, New South Wales). The fluorescence stain 4',6-diamidine-2-phenylindole' dihydrochloride (DAPI) was obtained from Sigma-Aldrich (Castle Hill, New South Wales). Full length PEPP2 in TOPO pCR2.1 was a generous gift from Dario R Alessi, MRC protein phosphorylation Unit, University of Dundee, Scotland UK. The PEPP2 ORF was subcloned into pEGFP-C2 (Clontech). COS1 cells and HEK-239T cells used in immunofluorescence were transfected with FuGene transfection reagent (Roche Diagnostic, Australia) using the manufacturer's recommended protocol.

Immunofluorescence analysis

Preparations of the various GFP-tagged expression constructs were made using the Qiagen Midi kit (Qiagen, Clifton Hill, Victoria). Two picomoles (approximately 1 microgram) of each construct were transfected into cultured COS1 cells on 6-well plates using FuGene transfection reagent (Roche Diagnostics Australia). Transfected cells were grown on coverslips in DMEM plus 10% FBS and fixed 24 hours post-transfection as previously described (Cox *et al,* 2000). Cells were then incubated with an anti-α tubulin (Sigma) or an anti-EEA1 (BD) antibody, followed by incubation with

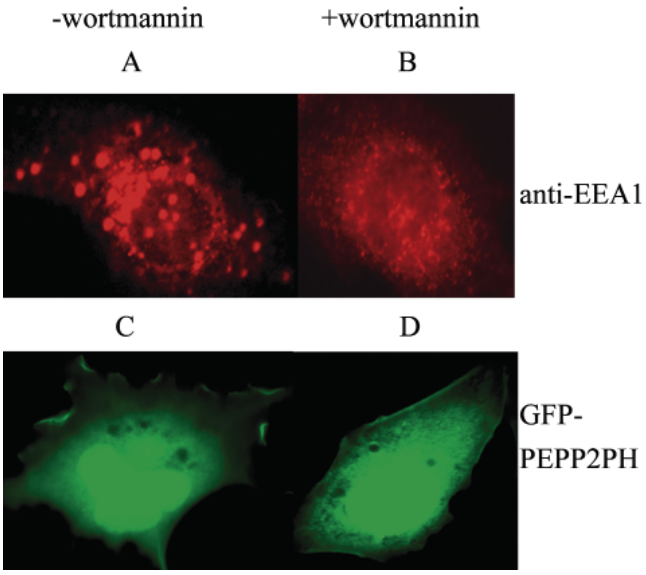

FIGURE 2. The membrane-association of the PH domain of PEPP2. The PH domain of PEPP2, expressed as a GFP fusion protein in COS1 cells (C and D), was observed on the plasma membrane as well as diffusely in both the cytoplasm and in the nucleus. The plasma membrane-association of GFP-PEPP2PH was unlikely to be mediated by PI3K product since the membrane-association remained unaffected after the treatment with wortmannin (D). Wortmannin inhibits PI3-kinase activity and reduces PI3K products, such as PI(3)P and PI(3,4,5)P3. Early endosome antigen 1 (EEA1), which associates with early endosomes (A) through interaction with PI(3)P, was redistributed into the cytoplasm (B) following wortmannin treatment.

an anti-mouse Texas Red-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, Maine). In all cases, nuclei were stained using the DNA-specific stain, DAPI. GFP and Texas Red fluorescence were visualised under appropriate wavelength light on an Olympus AX70 microscope. Images were captured using a Photometrics CE200A Camera Electronics Unit and processed using Photoshop 6.01 software (Adobe Systems Incorporated, San Jose, California).

Wortmannin treatment

Wortmannin was added to cell culture media (DMEM+ 10%FCS) at a final concentration of 100 nM (Simonsen *et al.*, 1998). Following addition, cells were cultured for a further 30 minutes before immunofluorescence analysis.

FRAP analysis for membrane microviscosity

Cells were grown on 90 mm dishes, which were modified with 15 mm diameter holes drilled at the centre of the base and sealed with coverslips. The plasma membrane was labelled with 8.3 µg/ml $DiIC_{16}$ at 4°C for 15 min and the medium replaced with serum-free DMEM plus 25 mM HEPES before imaging (Ghosh *et al.*, 2002). Photobleaching of approximately a micron diameter region on the plasma membrane was made with short pulses from a 543nm HeNe laser beam at 100% power and the fluorescence recovery monitored by scanning the bleached area with an attenuated beam of 20% power using a BioRad Radiance 2100 confocal microscope.

GST fusion protein expression and purification

An overnight culture of BL21 cells carrying the GST fusion protein expressing construct was diluted 100 fold into fresh L-broth +ampicillin and incubated at 37°C with vigorous shaking until the A_{600} reached 1.0. Cells were then induced with 0.2mM IPTG at 37°C for additional 3 hours, after which they were pelleted by centrifugation at 7,700 x g for 10 min and resuspended in 1/100 volume of STE plus 5mM DTT and 1.5% Sarkosyl. Collected cells were subsequently lysed on ice by sonication in short bursts. The insoluble fraction was separated by centrifugation at 12,000 x g for 5 min. 1/50 volume 50% slurry of Glutathione Sepharose 4B was then added to the remaining supernatant and incubated with gentle agitation at room temperature for 30 min. The Glutathione Sepharose 4B matrix was then washed three times with 1x PBS. The bound GST or GST fusion protein was finally eluted with an equal volume of Glutathione Elution Buffer.

Results

The lipid binding specificity of the PH domain of PEPP2

PH-domain containing proteins function through their interaction with phosphoinositides. Although no experimental data has shown that PEPP2 interacts with phosphoinositides, the PH domain of a closely related PH-domain containing protein, PEPP1, demonstrated PI3P binding specificity in an

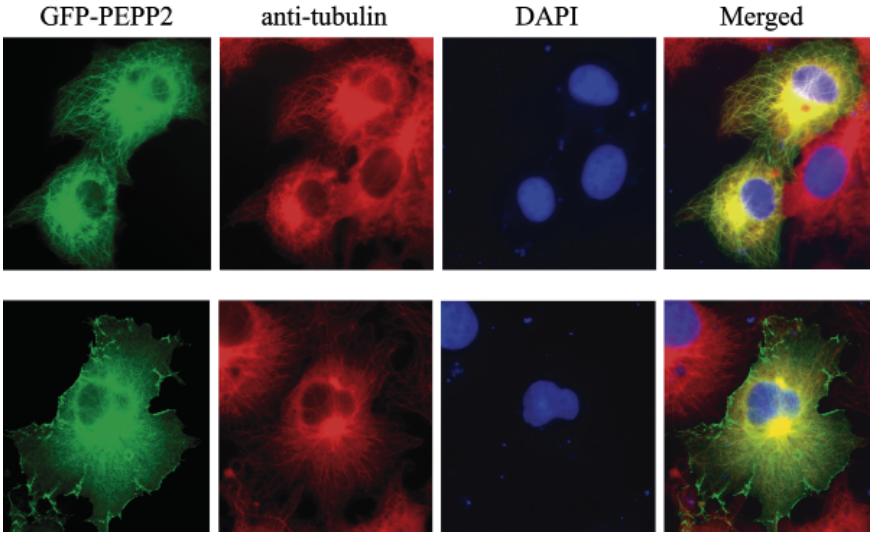

FIGURE 3. PEPP2 associates with both the microtubule cytoskeleton and plasma membrane. Cellular localization of overexpressed GFP-PEPP2 fusion protein in COS1 cells. Microtubule association (top panel) and dual microtubule/plasma membrane association (bottom panel) of GFP-PEPP2 was observed. Microtubules were stained with monoclonal anti-α-tubulin and a Texas-red-conjugated secondary antibody. Nuclei were stained with DAPI (blue). Enriched GFP-PEPP2 in the perinuclear region, in the proximity of the microtubule organization center, was observed with both microtubule and membrane-associated distributions.

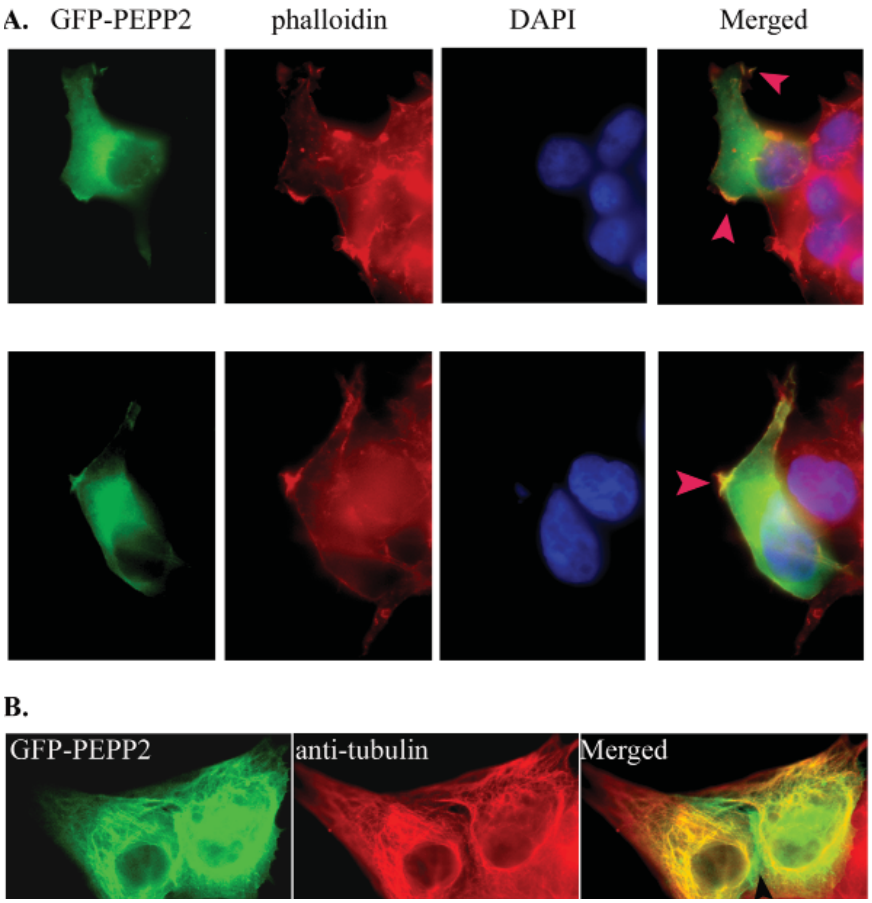

FIGURE 4. The non-uniform distribution of GFP-PEPP2 on the plasma membrane. A. In confluent HEK-293T cells transiently expressing GFP-PEPP2, scratches were introduced to promote cell migration in wound healing. The actin filaments were stained with phalloidin (red). A non-uniform distribution of GFP-PEPP2 on the plasma membrane at the leading edge of migrating cells was observed using direct immunofluorescence. The membrane-associated GFP-PEPP2 colocalized with the actin polymerization site (yellow), indicated by the arrowhead on merged images. Nuclei in these cells were stained with DAPI (blue). B. Microtubule-associated GFP-PEPP2 and membrane-associated GFP-PEPP2 enriched at site of cell-cell contact (indicated by arrowhead) were observed in epithelial MDCK cells. Microtubules were stained with anti-α-tubulin and a Texas-red-conjugated secondary antibody.

in vitro lipid binding assay. The coding sequence of the PH domain of PEPP2 was cloned into pGEX-4T-2 vector. The GST-PH (from aa130 to aa289) was expressed in BL21 cells and purified from cell lysates using Glutathione Sepharose 4B. Approximately 0.5ug/ml GST or GST-PEPP2PH were used in the protein-lipid overlay assay using the PIP strips with the indicated phosphoinositides of 100 pmol each. The strongest interactions were observed between the PH domain of PEPP2 and PI(5)P, PI(3)P and PI(3,5)P2, while weak interactions were also observed with the PI(4)P, phosphatidic acid (PA) and phosphatidylserine (PS) (Fig. 1). Control GST protein didn't bind any of these lipids (not shown).

To verify its lipid binding ability, the PH domain was also expressed as a GFP-fusion protein in COS1 cells. Despite the diffusive distribution in the cytoplasm and in nuclei, a significant portion of GFP-PH localized to the plasma membrane (Figure 2). Furthermore, this plasma membrane association of the GFP-PH fusion protein was not affected following wortmannin treatment (a PI3-kinase inhibitor). In contrast, the distribution of EEA1 (recruited onto the membrane of early endosomes through association with PI3P) was changed as expected after the same treatment (Fig. 2). These findings indicate that the PH domain of PEPP2 was unlikely to have PI3P or PI(3,5)P2 as its membrane binding partner *in vivo.*

The cellular localization of PEPP2

To verify that the membrane association of the GFP-PH fusion reflects that of the full length PEPP2 protein, PEPP2 was also expressed as a GFP fusion protein in COS1 cells. GFP-PEPP2 was found to show a variable localization, with distribution mainly on the plasma membrane but also on mi-

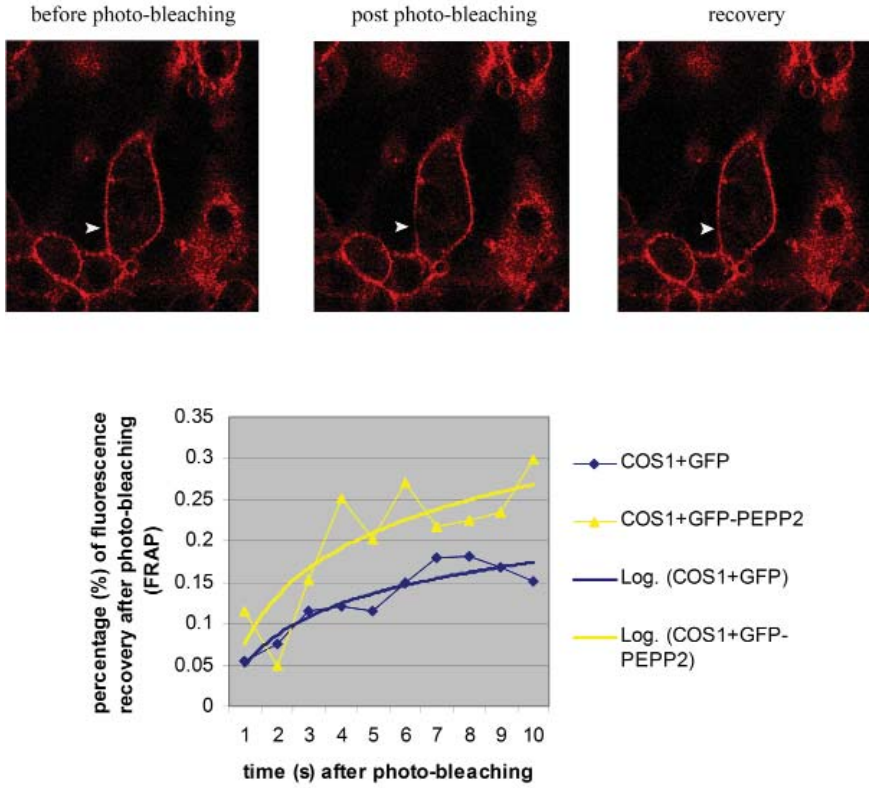

FIGURE 5. PEPP2 alters the lateral diffusion of the plasma membrane as measured by FRAP (fluorescence recovery after photo-bleaching). **A.** The plasma membrane of live COS1 cells was labelled with fluorescent $DiIC_{16}$ (3) (red), which is an analogue of membrane lipids. A well defined region on the plasma membrane was bleached, as indicated by arrowheads on the consecutive images above. The intensity of fluorescence recovery in the bleached area, which represented the lateral diffusion of membrane, was monitored and imaged over the first 10 seconds post-bleaching. **B.** Plasma membrane microviscosity of COS1 cells is increased by overexpressed PEPP2. The average recovery of fluorescence intensity (%) obtained from individual cells is presented in the graph (n=8). Trend lines are also shown.

crotubules (Fig. 3). Notably, a significantly increased amount of fluorescence of membrane associated GFP-PEPP2 was observed at cell-cell contacts (in both mesenchymal COS1 cells and polarized MDCK cells) (Fig. 4B). When the transiently transfected HEK-293T cells were induced to migrate by 'scratching' the confluent cell monolayer, a non-uniform distribution of membrane-associated GFP-PEPP2 was found, with enrichment at the actin-polymerization site at the leading edge of the migrating cells (Fig. 4A).

PEPP2 affects membrane microviscosity

Given that a significant proportion of PEPP2 is located on the plasma membrane, the effect of PEPP2 on a general plasma membrane property, membrane microviscosity, was investigated by fluorescence recovery after photo-bleaching (FRAP). FRAP has been shown to be easy and effective for measuring the lateral diffusion of membranes, which is inversely proportional to membrane viscosity (Ghosh *et al.*, 2002; Vereb *et al.*, 2003). Furthermore, the diffusion rate in a lipid bilayer is expected to be independent of the size of the bleached area (Vereb *et al.*, 2003). The plasma membrane of live untransfected COS1 cells or COS1 cells overexpressing GFP-PEPP2 was initially fluorescently labelled using $DiIC_{16}$(3). A small, defined area (μm range) was then photo-bleached and the recovery of fluorescence in this spot was subsequently monitored and imaged by scanning with a confocal microscope (Fig. 5a). The fluorescence recovery was monitored every second for 10 seconds post photo-bleaching. Compared with the control COS1 cells, the fluorescence recovery was enhanced in COS1 cells overexpressing PEPP2, indicating a potential role for PEPP2 in regulating membrane function (Fig. 5b).

Discussion

The physical characteristics of microtubules and their interaction with other microfilaments and membranes through cross-linking proteins inextricably link this cytoskeletal network to a vast array of cellular processes.

Cross-talk between membranes and the microtubule cytoskeleton is critical for signal transduction, intracellular trafficking and organelle positioning (Mayer and Jurgens, 2002). Membrane-cytoskeleton linkages can be established via both protein-protein and protein-lipid interactions. Known cross-linking proteins include those of the CLIP family, microtubule motors, and various microtubule-associated proteins (MAPs). These proteins can establish connections between microtubules and subcellular membranes, and serve as multiple docking sites for both organelle localization and vesicle trafficking (Schroer, 2000). A large number of cross-linking proteins also utilize protein-lipids interactions to help them anchor to membranes (Schroer, 2000).

PEPP2/PLEKHA5 is a novel PH domain-containing protein, consisting of 1060 amino acids and having two WW domains at its N-terminus. We show here that the plasma membrane-association of PEPP2 is likely directed by the protein-lipid interaction between the PH domain and phosphoinositides since the overexpressed PH domain-GFP fusion exhibited prominent association with the plasma membrane. Although interactions between the PEPP2 PH domain and a number of different phosphoinositides (PI5P, PI(3,5)P2 and PI4P) were found in the *in vitro* lipid interaction assay, the binding of PI-3-phosphate derivatives *in vivo* is unlikely since the membrane associated PH domain of PEPP2 remained unaffected following treatment with wortmannin, a PI3K inhibitor.

A non-uniform distribution of PEPP2 was observed on plasma membrane ruffles, where actin polymerization occurs. Actin polymerization and microtubule protrusion at the leading edge of motile cells is required for directed cell migration (Nabi, 1999). Different phosphoinositides, including PI(3,4,5)P_3, PI(3,4)P_2 and PI(4,5)P_2, have been indicated in regulating actin dynamics (Insall and Weiner, 2001). For instance, PI(3,4,5)P3 serves as an instructive signal for stimulus-induced actin polymerization via activation of the guanine nucleotide exchange factors (GEFs) of the Rho family (Insall and Weiner, 2001). Clustering of PI(4,5)P_2 at the actin nucleation site on the plasma membrane has also been shown (Tall *et al.*, 2000). PI(4,5)P2 can be generated by PI(4)P-5-kinases or PI(5)-4-kinases from both PI(4)P and PI(5)P (van den Bout & Divecha, 2009), which we have shown are able to bind to the PH domain of PEPP2. Although the PH domain of PEPP2 showed much higher affinity for PI5P than PI4P in the in vitro lipid binding assay, the local concentration of each phosphoinositide and the interaction of PEPP2 with other proteins may be as, or more, important than the lipid binding affinity in directing the protein-lipid interaction under physiological conditions. For example, it is possible that the interaction between other proteins and the WW-domain or other uncharacterized regions of PEPP2 also helps to specify the membrane targeting and hence the cellular function of PEPP2 (Chang *et al.,* 2010). Such a mechanism is generally used by PH-domain-containing proteins with no clear ligand specificity and low binding affinity (Lemmon *et al.,* 2002).

The observation that PEPP2 concentrated at the actin nucleation site raises the possibility of its influence on actin polymerization. The polymerization of actin and microtubules are coordinated by Rho family GTPases, such as Rho and Rac1. The ruffled leading lamellipodium of migrating cells reflects the underlying polarized organization of the cytoskeleton (Rodriguez *et al.,* 2003). The structural linkage between microtubules and actin also orients the plus end of microtubules towards the leading edge along moving actin bundles (Gundersen and Bretscher, 2003). Therefore, PEPP2 may be involved in coupling the reorganization of microtubules with actin polymerization in directing cell migration.

Both phospholipids and the cytoskeleton (as well as cholesterol and integral membrane proteins) affect the viscosity of membranes - viscosity being the reciprocal of membrane fluidity (Vereb *et al.,* 2003). Given the membrane-association of PEPP2 and its possible role in regulating actin polymerization, the potential influence of PEPP2 on membrane microviscosity was investigated using FRAP. A notable increase in the lateral movement of the plasma membrane was observed in cells overexpressing GFP-PEPP2. The relationship between membrane microviscosity and cell motility is biphasic. Increased cell motility has been seen with increased microviscosity to a threshold, beyond which resulted in significantly decreased motility (Ghosh *et al.,* 2002). The potential impact of PEPP2 on cell motility due to the altered membrane viscosity needs further investigation.

We have shown here the lipid binding specificity of the PEPP2 PH domain and likely phosphoinositide interactions of full length PEPP2 *in vivo.* Overexpressed wild type PEPP2 displayed an interesting dual subcellular localization, with predominant localization on the plasma membrane and variable association with microtubules. This pattern of cellular distribution of PEPP2 and data from FRAP assays suggests a role for this novel PH domain containing protein in participating in cellular events involving these two distinct cellular compartments, such as cell migration and membrane trafficking.

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