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Phosphatidylinositol 5-phosphate is a second messenger important for cell migration

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We recently showed that production of phosphatidylinositol 5-phosphate (PtdIns5P or PI5P) upon growth factor stimulation is important for cell migration. However, it was not entirely clear if PI5P itself could be a second messenger in cell migration, or, if it was rather an intermediate for the production of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2 or PI(4,5)P_2). Indeed, PI5P can be converted to PI(4,5)P_2 by type II PIP4 kinases (PIP4K2s). We therefore decided to knock down PIP4K2α by siRNA to test if further conversion of PI5P to PI(4,5)P_2 is important for cell migration. Even though we obtained an efficient knockdown of PIP4K2α in BJ human fibroblasts, we did not observe any change in cell velocity. Conversely, ectopic overexpression of PIP4K2α would consume PI5P to produce PI(4,5)P_2 and we found that overexpressing PIP4K2α decreased cell migration speed. Taken together, the data clearly indicate that it is PI5P, and not PI(4,5)P_2 produced from PI5P, that is the crucial signaling molecule in cell migration. We conclude, therefore, that PI5P is a true second messenger important for cell migration.

Introduction

In a recent publication, we showed that growth factor signaling through the receptor tyrosine kinase FGFR1, increased cellular levels of PI5P and concomitantly cell migration.[1] PI5P production in this case, was due to a phosphoinositide metabolic loop consisting of three enzymes; phosphatidylinositol-3-kinase class III (PI3KC3), PIKfyve and MTMR3. In the first step of this pathway, PI3KC3 produce phosphatidylinositol 3-phosphate (PI3P) from phosphatidylinositol (PI) by phosphorylating the inositol headgroup on the 3'-position. PI3P can also possibly be produced by class II phosphatidylinositol-3-kinase.[2,3] Then PIKfyve phosphorylates PI3P on the 5'-position, thereby producing PI(3,5)P_2. Finally, the lipid phosphatase MTMR3 dephosphorylates the 3'-position resulting in the production of PI5P (**Fig. 1**). Importantly, we also demonstrated that in PIKfyve and MTMR3 knockdown cells, exogenously added PI5P could rescue cell migration arguing that their product, PI5P, is the relevant signaling molecule. Further experiments in *Drosophila melanogaster* showed that this pathway operates in vivo and is conserved among different species.

However, even though we firmly established a role for PI5P in cell migration, the possibility existed that PI5P could be an intermediate for the further production of PI(4,5)P_2. A family of enzymes catalyzing this reaction has been characterized in human cells.[4] There exist three isoforms of these enzymes: phosphatidylinositol-4-phosphate 5-kinase II α, β and γ (PIP4K2α, PIP4K2β and PIP4K2γ, respectively). Interestingly, PI(4,5)P_2 plays well-established roles in cell migration[5] and the further conversion of PI5P to PI(4,5)P_2 could explain the observed effects on cell migration. It is believed that the bulk of PI(4,5)P_2 in cells is made from phosphatidylinositol 4-phosphate (PtdIns4P or PI4P), which is much more abundant than PI5P (~100 times more).[4] However, it is a possibility that distinct pools of PI(4,5)P_2 made from PI5P could be important in certain locations within the cell.[6]

PIP4K2β is found mainly in the nucleus of cells,[7] while PIP4K2α and PIP4K2γ are predominantly found in the cytoplasm.[4,8] PIP4K2α is significantly more active than the two other isoforms and it has been suggested that the β- and γ-isoforms can dimerize with the α-form.[4] The role of the β- and γ-isoforms may therefore rather be to target the high enzymatic activity of the α-form to certain locations.

Interestingly, Wilcox and Hinchliffe (2008) showed that enhanced tyrosine phosphorylation in cells resulted in increased PI5P production,[9] which could be analogous to our observed increase in PI5P levels when cells were stimulated with the growth factor FGF1, that also causes enhanced tyrosine kinase signaling.[1] Interestingly, overexpression of PIP4K2α inhibited PI5P production in this case.[9] Furthermore, only siRNA depletion of PIP4K2α, and not PIP4K2β or PIP4K2γ, increased PI5P levels even more when tyrosine kinase signaling was activated. It is therefore likely that PIP4K2α is the relevant kinase in the regulation of non-nuclear PI5P levels.

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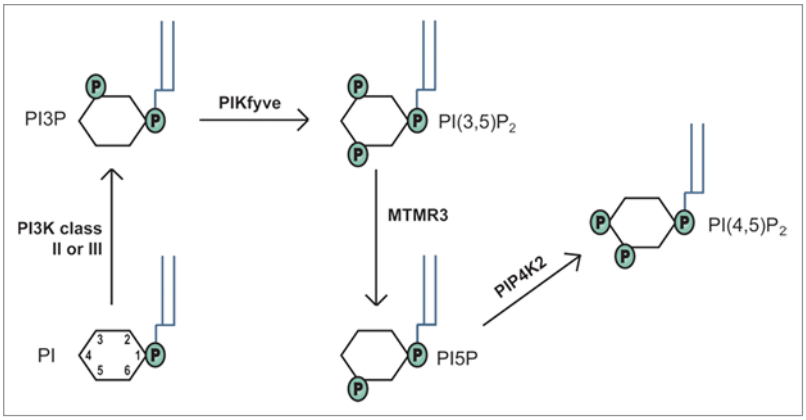

Figure 1. Schematic representation of the pathway for $PI(4,5)P_2$ synthesis via PI5P. The lipid part of the phosphoinositides is shown in blue, the inositol headgroup in black and the phosphorylated sites in green.

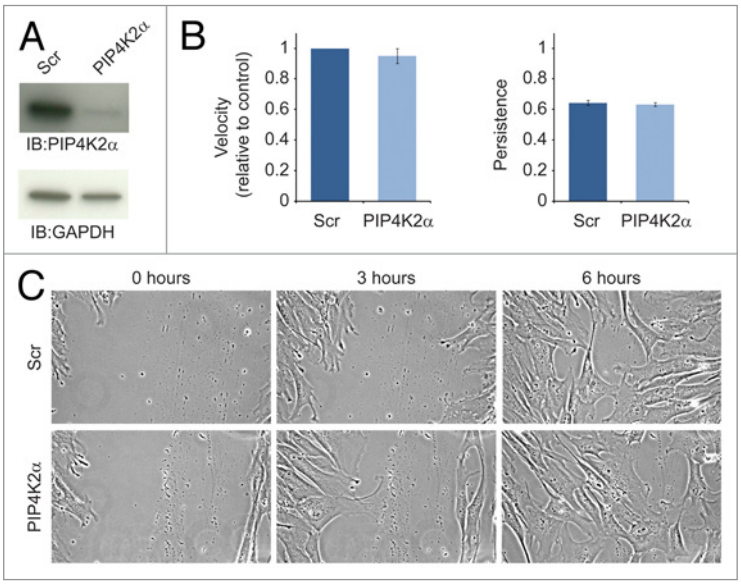

Figure 2. Depletion of PIP4K2α does not affect cell velocity or persistence. (**A**) BJ cells were transfected with 50 nM non-targeting (scr) or PIP4K2α-targeting siRNA oligos. 48 h after the first transfection, the cells were re-transfected. The cells were lysed 48 h after the last transfection and the lysates were analyzed by SDS-PAGE and western blotting. One representative western blot is shown. (**B**) BJ cells transfected as described above were scratched and treated with FGF1 to facilitate cell migration. The cells were then recorded for 6 h and the mean velocity and persistence for the last 3 h were quantified as described in materials and methods. The graph represents the mean ± SEM of three independent experiments. In total, 142 cells were analyzed for scr and 110 for PIP4K2α depleted cells. (**C**) Representative images of BJ cells treated as described above at 0, 3, and 6 h after wound scratch.

As PIP4K2α is the most active form of the three type II PIP4-kinases, it was used for the investigations in this study. We have here studied the effect of manipulating PIP4K2α expression levels on cell migration. We found that depletion of PIP4K2α does not change the velocity or persistence of cells, while overexpressing a PIP4K2α-GFP fusion protein decreases velocity.

Results and Discussion

To test if cellular levels of PIP4K2α influence the velocity of BJ human fibroblasts, we depleted cells for PIP4K2α using a specific siRNA oligonucleotide targeting PIP4K2α. To ensure an efficient knockdown, cells were first transfected and then re-transfected after 48 h and the experiments were performed 96 h after the first transfection. Efficient knockdown was confirmed by western blotting (**Fig. 2A**) and by real time PCR (PIP4K2α mRNA level was reduced to 5.3% of control 48 h after transfection). The role of PIP4K2α in BJ fibroblast cell migration was then studied by live-cell imaging of FGF1-stimulated BJ fibroblasts in a wound healing assay. Knockdown of PIP4K2α did not alter the velocity of the cells nor their persistence (**Fig. 2B**) and both PIP4K2α knockdown cells and cells transfected with non-targeting control siRNA (scr) were able to migrate into the wound to a similar extent (**Fig. 2C**). The data indicates that the pool of $PI(4,5)P_2$ produced from PI5P by PIP4K2α is not crucial for cell migration.

Next, we wanted to test if increasing the levels of PIP4K2α in cells would affect cell migration. An increased level of PIP4K2α could consume more PI5P to produce $PI(4,5)P_2$ and therefore lead to lower levels of PI5P and higher levels of $PI(4,5)P_2$.

We ectopically expressed a PIP4K2α-GFP fusion protein in BJ cells and measured cell motility by live-cell imaging. Due to low transfection efficiency of BJ cells, imaging of random cell migration of subconfluent cells rather than the wound healing assay was performed in this case. Cells expressing PIP4K2α were identified by fluorescence microscopy of the GFP signal, tracked over time and their velocity was then compared with non-transfected cells. Interestingly, we observed a reduction in cell velocity in cells expressing PIP4K2α (**Fig. 3A**), while expression of GFP alone in cells did not change cell velocity (**Fig. 3B**). The reduced migratory potential of cells expressing PIP4K2α, an enzyme producing $PI(4,5)P_2$ from PI5P, indicates that PI5P itself could be a second messenger in cell migration. This is also in agreement with our previous finding that ectopic expression of the bacterial enzyme IpgD, which converts $PI(4,5)P_2$ into PI5P, at least partly rescues the migratory defects in MTMR3 and PIKfyve siRNA-treated cells.[1,10]

Our previous study and the data presented here, clearly show that PI5P plays a role as a second messenger in cell migration. The current data argue against the possibility that PI5P could merely be an intermediate for the further production of $PI(4,5)P_2$, an already known regulator of cell migration.[5] In our cells, depletion of PIP4K2α did not alter cell velocity, indicating that the production of $PI(4,5)P_2$ from PI5P is not required for cell migration. Rather, it is possible that PIP4K2α is involved in attenuating signaling from PI5P by reducing cellular PI5P levels.[4] Depletion of PIP4K2α could potentially lead to an

increase or a stabilization of PI5P levels in cells. However, this was in our cells not enough to drive the migratory potential of cells beyond that of FGF1-stimulation. Even though depletion of PIP4K2α did not increase cell migration velocity in our cells, the conversion of PI5P to $PI(4,5)P_2$ could in certain circumstances act to decrease cell movement, for instance, when a certain migratory trajectory is completed during development or wound healing. This possibility is strengthened by our present observation that overexpression of PIP4K2α reduced cell migration, indicating that the pool of PI5P produced by FGF1-stimulation is accessible for conversion by PIP4K2α. Wilcox and Hinchliffe (2008) also found that ectopically overexpressing PIP4K2α reduced the levels of PI5P generated by pervanadate treatment.[9] However, Hinchliffe and colleagues earlier observed that basal PI5P levels were not affected by overexpression of PIP4K2α.[11] Taken together, these studies suggest that there may be multiple pools of PI5P in cells where some are not accessible to ectopically overexpressed PIP4K2α.

We recently published data suggesting that PI5P may regulate actin dynamics in cell migration.[1] Other studies have also implicated actin cytoskeleton remodeling downstream of PI5P. Interestingly, Sbrissa and colleagues found that microinjection of PI5P into cells caused F-actin stress fiber breakdown.[12] Other phosphoinositides did not cause this effect, demonstrating a remarkable specificity for PI5P. Along the same lines, Niebuhr et al. found that ectopically overexpressing the bacterial enzyme IpgD, which converts $PI(4,5)P_2$ into PI5P had dramatic effects on cell morphology and the actin cytoskeleton.[9,13]

We, therefore, favor the hypothesis that PI5P recruits effector proteins to specific membrane compartments during cell migration and it is likely that such an effector could be a molecule involved in cytoskeleton remodeling. Possible candidates could be activators of the Rho GTPase family. It is also possible that PI5P binding proteins with other functions can play roles during cell migration. For instance, PI3P recruits many different proteins to early endosomes to orchestrate endosomal transport and functions.[14] In the case of PI3P, many specific effector proteins have been found.[14] Proteins with FYVE domains (e.g., EEA1, HRS or PIKfyve) or PX domains (e.g., sorting nexins), often bind specifically to PI3P. Unfortunately, there is much less known about PI5P binding proteins. Several proteins have been found to bind PI5P[15-17] but often they also bind to other phosphoinositides, like PI3P and PI4P. As PI5P is found in small quantities compared with other phosphoinositides this has made it difficult to develop specific probes to localize PI5P within the cell. An important goal will therefore be to identify PI5P binding proteins that can be used to determine the locations of PI5P in cells and to explain the effects of signaling-induced PI5P production.

Material and Methods

Antibodies and reagents. The rabbit anti-PIP4K2α antibody was purchased from Cell Signaling. The mouse anti-GAPDH-HRP (Ab9482) loading control was from Abcam. FGF1 was prepared as previously described.[18] Heparin was from Sigma-Aldrich.

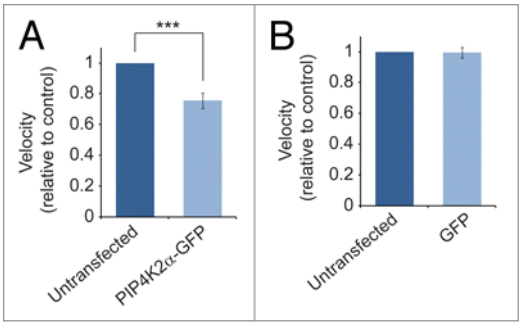

Figure 3. Expression of PIP4K2α reduces cell velocity. Subconfluent BJ cells transfected with PIP4K2α-GFP (**A**) or GFP (**B**), were treated with FGF1 to facilitate cell migration. The cells were then recorded for 8 h and the mean velocity for the last 5 h were quantified as described in materials and methods. The graph represents the mean ± SEM of five independent experiments. In total, 107 PIP4K2α-GFP expressing cells and 117 untransfected cells were analyzed in (**A**) and 132 GFP expressing cells and 146 untransfected cells were analyzed in (**B**). ***$p \leq 0.001$, ONE-WAY ANOVA Tukey's test.

Cell culture. The human normal foreskin fibroblast cell line BJ was cultured in Quantum 333 For Fibroblasts from PAA Laboratories GmbH, supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37°C. Cells were seeded into tissue culture plates the day preceding the start of the experiments.

Plasmids and siRNA oligos. Silencer® Select non-targeting negative control siRNA oligos and siRNA oligos targeting *PIP4K2A* (sense siRNA: 5'-CCGGCUUAAUGUUGAUGGAtt-3') were purchased from Ambion, Life Technologies. GFP-tagged ORF clone of human *PIP4K2A* was purchased from OriGene Technologies. pEGFP-N1 was from ClonTech.

Transfection. siRNA transfection were performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Life Technologies) according to the manufacturer's protocol. The cells were re-transfected after 48 h and the experiments were performed 48 h after the last transfection. Transient expression of different constructs was performed by transfecting cells with plasmid DNA using Fugene 6 Transfection Reagent (Roche Diagnostics) according to the manufacturer's protocol.

RNA isolation, cDNA synthesis and quantitative real-time polymerase reaction (qRT-PCR). Total RNA was isolated from cell lysate using RNeasy plus mini kit (Qiagen) and the QIAcube robot (Qiagen) according to the manufacturer's protocol. Then 0.5 μg of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen), cDNA template and the following QuantiTect primers (Qiagen); PIP4K2A (QT00075894) and Succinate dehydrogenase (SDHA) (QT00059486). The qRT-PCR was run and analyzed using the Lightcycler 480 (Roche Diagnostics). Cycling conditions were 5 min at 95°C followed by 45 cycles 10 sec at 95°C, 20 sec at 60°C and 10 sec at 72°C. Gene amplification was normalized to the expression of SDHA. Relative quantification was done with the LightCycler® 480 software.

Western blotting. Cells were lysed in lysis buffer (0.1 M NaCl, 10 mM Na_2PO_4, 1% triton X-100, and 1 mM EDTA, pH 7.4) supplemented with protease inhibitors (Roche Diagnostics). The lysates were then loaded for SDS-PAGE (4-20% gradient) and afterwards transferred to PVDF membrane (Bio-Rad) for western blotting. Blots were developed with the Amersham ECL Plus western blotting Detection System (GE Healthcare).

Time-lapse live-cell imaging and cell tracking. Confluent BJ cells plated on Glass Bottom Dishes (MatTek Corporation) were observed with a BioStation IM Live Cell Recorder (Nikon Instruments Inc.). Image acquisition was done every 10 min. Cells were stimulated with FGF1 (100 ng/ml) and heparin (50 U/ml) in all experiments. For wound healing experiments, confluent cells were scratched with a 10 μl tip prior to live-cell imaging. Images were analyzed with Image J software with Manual Tracking and Chemotaxis and Migration Tool (ibidi GmbH) plugins. Persistence was calculated by dividing the Euclidean distance by the accumulated distance of the cell trajectories.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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