## RESEARCH COMMUNICATION

# Platelet-derived growth factor-induced phosphatidylinositol 3-kinase activation mediates actin rearrangements in fibroblasts

Matthias WYMANN* and Alexandre ARCARO

Institute of Biochemistry, University of Fribourg, Rue du Musée 5, CH-1700 Fribourg, Switzerland

Various agonist-induced cell responses in neutrophils and fibroblasts, such as chemotaxis and cytoskeletal rearrangements, have been shown to correlate with the synthesis of PtdIns(3,4,5)$P_3$; however, the significance of this rise in second messenger levels is not clear. We show here that wortmannin inhibits platelet-derived growth factor (PDGF)-mediated production of PtdIns(3,4,5)$P_3$ in human foreskin fibroblasts with an $IC_{50}$ of about 5 nM. A similar inhibition was observed in *in vitro* assays ($IC_{50} \sim 1$ nM) with phosphatidylinositol 3-kinase immunoprecipitated by antibodies directed against the 85 kDa subunit (p85). On the other hand, wortmannin did not affect PDGF-mediated phosphorylation of p85 as detected by immunoprecipitation with anti-phosphotyrosine antibodies, and did not dissociate the complex of p85 and the catalytic subunit (p110) of phosphatidylinositol 3-kinase. These results are consistent with a direct, specific inhibition of the enzyme by wortmannin at concentrations relevant for its previously reported effects on cellular responses. When stimulated with PDGF, human foreskin fibroblasts form circular structures of filamentous actin. Preincubation of these cells with wortmannin inhibits PDGF-mediated actin rearrangements, suggesting a need for PtdIns(3,4,5)$P_3$ formation as a signal for this cell response.

## INTRODUCTION

Agonist-induced formation of PtdIns(3,4,5)$P_3$ has recently received much attention, because it parallels a variety of cell responses [1–3], and the molecule has been proposed to constitute a novel second messenger. The regulatory subunit of PtdIns 3-kinase (p85) has been found to be associated with some protein tyrosine kinases, such as the polyoma middle-T/pp60$^{c\text{-}src}$ complex [4], and the activated receptors for epidermal growth factor [5] and platelet-derived growth factor (PDGF) [6].

PDGF plays a role in cell proliferation, chemotaxis, wound and tissue repair, fibrosis and vascular tension. The PDGF receptor is coupled to a complex signalling system involving proteins such as phospholipase C$\gamma$, p21$^{ras}$ GTPase-activating protein (rasGAP) and PtdIns 3-kinase [7]. Binding of PDGF dimers to their receptors initiates the association of two receptor chains, leading to the autophosphorylation of several tyrosine residues within the cytosolic domain [8]. When phosphorylated, Tyr-751 and Tyr-740 of the human PDGF-$\beta$ receptor seem to provide the docking sites for the two SH2 domains present on the 85 kDa subunit of PtdIns 3-kinase [4–6,9–12]. It has been shown that the rapid translocation to the growth factor receptor is followed by phosphorylation of p85 and activation of PtdIns 3-kinase [13,14]. Associated with p85, the 110 kDa catalytic subunit [15] is brought to the plasma membrane, where it gains access to its substrate PtdIns(4,5)$P_2$ and produces PtdIns(3,4,5)$P_3$ [2,3].

Motile and chemotactic responses demand reorganization of the cytoskeleton. When stimulated by PDGF, human foreskin fibroblasts partially break down stress fibres and produce circular membrane ruffles that are rich in filamentous actin [8]. Actin polymerization and depolymerization have been proposed to be controlled by proteins such as gelsolin [16,17] and profilin [18], whose affinities for actin are influenced by polyphosphoinositides. A role for PtdIns(4,5)$P_2$ in controlling actin rearrangements, however, is still a matter of debate: PtdIns(4,5)$P_2$ concentrations are lowered due to agonist-induced phospholipase C activation, and actin polymerization is unaffected by artificially lowered cytosolic calcium concentrations blocking phospholipase C-mediated PtdIns(4,5)$P_2$ hydrolysis [19]. Traynor-Kaplan et al. [20] suggested a role for PtdIns(3,4,5)$P_3$ in actin polymerization in neutrophils on the basis of similar progress curves of the two agonist-mediated responses.

Here we show that wortmannin inhibits PDGF-induced PtdIns 3-kinase activity without interfering with PDGF receptor tyrosine kinase signalling. Since wortmannin effectively blocks the formation of actin-rich membrane ruffles, this suggests that PtdIns(3,4,5)$P_3$ accumulation is essential for this cell response.

## MATERIALS AND METHODS

### Materials

Chemicals were purchased from sources described in [21]. Wortmannin was generously provided by Dr. T. G. Payne, Sandoz, Basel, Switzerland; PDGF-BB was a gift from Dr. M. Pech, F. Hoffmann-La Roche, Basel, Switzerland. NIH 3T3 and NIH 3T3$^{srcF527}$ fibroblasts were generously given by Dr. K. E. Amrein, F. Hoffmann-La Roche, Basel (see [22]).

### Cell culture and metabolic labelling

AG1523 fibroblasts (purchased from the Coriell Institute, Cambden, NJ, U.S.A.; passages 10–16) were grown to confluence for 4 days in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10% fetal calf serum, non-essential amino acids, pyruvate, penicillin and streptomycin). Serum-starved cells (16 h starvation) were washed twice with phosphate-free DMEM (GIBCO; without phosphate) for metabolic labelling before incubation with 500 $\mu$l of the same medium containing 0.2 mCi of [$^{32}$P]$P_i$ for 60 min at 37 °C. Cells were then washed twice in Hepes buffer (20 mM, pH 7.4, 138 mM NaCl, 4.6 mM KCl, 5 mM glucose, 2 mM $MgCl_2$ and 1 mM $CaCl_2$) and incubated in 500 $\mu$l of this buffer for 10 min at 37 °C. PDGF-BB was

Abbreviations used: PDGF, platelet-derived growth factor; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's medium; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine.

* To whom correspondence should be addressed.

subsequently added in 50 $\mu$l of Hepes buffer; 1 mg/ml fatty acid-free BSA was also added to give a final concentration of 100 ng/ml (unless stated otherwise).

### Lipid extraction and analysis

The reaction was stopped by the addition of 1.9 ml of chloroform/methanol (1:2, v/v) to cell layers, and lipids were extracted according to Hawkins et al. [2], before they were dried for deacylation [23]. Deacylated lipids were separated on the Partisil SAX column [21]. Retention times were compared with those of [$^{32}$P]glycerophosphoIns3*P* and [$^{32}$P]glycerophospho-Ins(3,4,5)$P_3$ standards produced with purified PtdIns 3-kinase (a generous gift from Dr. C. L. Carpenter, Harvard Medical School, Boston, MA, U.S.A.), and those of [$^3$H]Ins(1,4,5)$P_3$ and [$^3$H]Ins(1,3,4,5)$P_4$ standards (NEN).

### PtdIns 3-kinase assays in immunoprecipitations

Confluent AG1523 cells from five 10 cm Petri dishes were lysed as described elsewhere [24] and PtdIns 3-kinase present in the lysate was precipitated using anti-p85 antiserum (UBI). Immobilized PtdIns 3-kinase was then equally split to be incubated with wortmannin. [$^{32}$P]PtdIns3*P* formation was assayed essentially according to Kaplan et al. [13]. Radioactivity was quantified on a t.l.c. linear analyser (Berthold LB 2842). The identity of PtdIns3*P* was checked by deacylation and h.p.l.c. separation as described above.

### Tyrosine phosphorylation of p85 and association with p110

Resting or stimulated serum-starved cells were lysed separately and immunoprecipitated with anti-phosphotyrosine antibodies (UBI) essentially as described [24]. Immunoprecipitates were applied to 7.5–15% gradient SDS/PAGE gels, transferred to poly(vinylidene difluoride) membranes (Millipore) and PtdIns 3-kinase was detected with rabbit anti-p85 antiserum as indicated by the manufacturer (UBI) using enhanced chemiluminescence (ECL; Amersham).

To test the integrity of the p85/p110 complex in the presence of wortmannin, PtdIns 3-kinase was immunoprecipitated with anti-p85 antiserum (UBI) and exposed to wortmannin or buffer [with 0.1% dimethyl sulphoxide (DMSO)] only. Immobilized p110 was detected by immunoblotting using affinity-purified rabbit antibodies against a 21-mer peptide of the C-terminal part of p110 [15]. Subsequent to ECL detection, antibodies were stripped from the membranes (0.1 M $\beta$-mercaptoethanol, 63 mM Tris, 2% SDS, pH 6.7, for 30 min at 50 °C); the membranes were then reprobed for the presence of p85.

### Staining for filamentous actin

Cells grown on cover glasses were incubated with wortmannin and PDGF, washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, pH 7.4) and then stained (4% para-formaldehyde, 100 $\mu$g of lysophosphatidylcholine/ml and 0.33 $\mu$M fluorescein–phalloidin in PBS, overnight at 4 °C [25]).

## RESULTS AND DISCUSSION

We have studied the PDGF-BB-induced formation of PtdIns(3,4,5)$P_3$ in human AG1523 foreskin fibroblasts, which have been well characterized with regard to their content of PDGF-$\beta$ receptors [8] and binding of PtdIns 3-kinase to the autophosphorylated receptor [26]. When challenged with PDGF, AG1523 cells metabolically labelled with [$^{32}$P]$P_i$ rapidly responded with the production of PtdIns(3,4,5)$P_3$ (results not shown), similar to the response described in detail for smooth muscle [3] and Swiss 3T3 mouse fibroblasts [2]. When cells were preincubated with 100 nM wortmannin, the PDGF-mediated formation of [$^{32}$P]PtdIns(3,4,5)$P_3$ was totally abolished (Figure 1a). The inhibitory action of wortmannin was found to be dose-dependent, with an $IC_{50}$ of about 5 nM (Figure 1b).

To investigate whether the inhibition of PtdIns(3,4,5)$P_3$ production was due to an interaction of wortmannin with PtdIns 3-kinase or to some interference with the activation of the enzyme by the growth factor receptor, we immunoprecipitated the PtdIns 3-kinase complex from unstimulated cells with antibodies directed against its p85 subunit. PtdIns 3-kinase immobilized on Protein A–Sepharose showed an even greater sensitivity towards wortmannin (Figures 1b and 1c; $IC_{50} \sim 1$ nM) than the activity in the cellular system. Because *in vitro* synthesis of [$^{32}$P]PtdIns3*P* by PtdIns 3-kinase (from PtdIns and [$\gamma$-$^{32}$P]ATP) does not depend on any activation step, this demonstrates the direct action of wortmannin on the enzyme. Since Hiles et al. [15] have shown that the recombinant p110 subunit of PtdIns 3-kinase

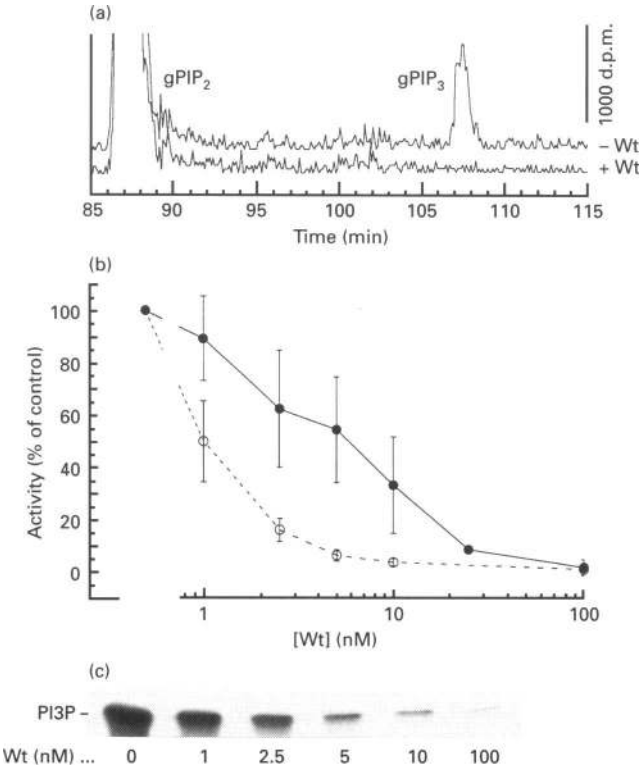


**Figure 1 Inhibition of PtdIns 3-kinase activity by increasing concentrations of wortmannin (Wt)**

(**a**) H.p.l.c. traces of deacylated lipids derived from $^{32}$P-labelled AG1523 cells stimulated with PDGF for 2 min. The upper trace (— Wt), displaying the presence of glycerophosphoIns(3,4,5)$P_3$ (gPIP$_3$), was obtained from cells kept in Hepes buffer (with 0.1% DMSO) at 37 °C for 15 min prior to stimulation. The lower trace (+Wt) represents results from cells pretreated with 100 nM wortmannin for the same period. Detected radioactivity is plotted as d.p.m. versus retention time. (**b**) Inhibition by wortmannin of PDGF-mediated PtdIns(3,4,5)$P_3$ formation in fibroblasts (●, $n = 5$, means ± S.D.) and PtdIns3*P* production in PtdIns 3-kinase immunoprecipitates (○, $n = 4$). $^{32}$P-labelled cells and immunoprecipitates were incubated for 15 min at 37 °C with the indicated concentrations of wortmannin. Cells were subsequently stimulated for 2 min with 100 ng/ml PDGF and immunoprecipitates were supplemented with the substrates for [$^{32}$P]PtdIns3*P* production. Data are plotted as percentages of control values (activity in the presence of 0.1% DMSO only). (**c**) Autoradiography of a representative t.l.c. showing the formation of PtdIns3*P* (PI3P) by immunoprecipitated PtdIns 3-kinase as a function of the amount of wortmannin present during preincubation.

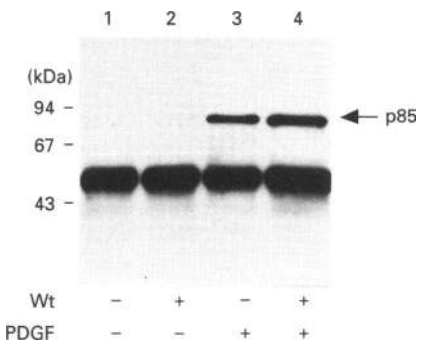


**Figure 2 Effect of wortmannin on PDGF-induced tyrosine phosphorylation of the regulatory subunit (p85) of PtdIns 3-kinase**

Serum-starved AG1523 fibroblasts were incubated for 30 min at 37 °C in DMEM containing DMSO (0.1%, lanes 1 and 3) or 1 $\mu$M wortmannin (Wt; lanes 2 and 4). PDGF (100 ng/ml; lanes 3 and 4) or vehicle only (lanes 1 and 2) was then added and the cells were kept for a further 10 min at 37 °C before lysis. Phosphotyrosine-containing proteins were immunoprecipitated and subjected to immunoblotting with anti-p85 antibodies.

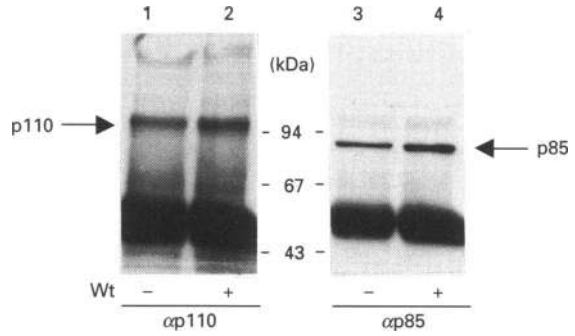


**Figure 3 Integrity of the p85/p110 complex of PtdIns 3-kinase in the presence of wortmannin**

PtdIns 3-kinase was immunoprecipitated from AG1523 cells using anti-p85 antiserum and subsequently incubated without (0.1% DMSO only) or with 1 $\mu$M wortmannin (Wt) at 37 °C for 15 min. Immobilized proteins were submitted to SDS/PAGE and immunoblotting with affinity-purified anti-p110 antibodies (lanes 1 and 2). Antibodies were then removed (see the Materials and methods section) and the same membranes were re-exposed to anti-p85 antibodies (lanes 3 and 4).

displays catalytic activity in the absence of p85, wortmannin is more likely to interact with p110 than with the regulatory subunit; this conclusion is also supported by the results below.

To exclude possible effects of wortmannin on PDGF receptor activation, agonist-mediated tyrosine phosphorylation of the p85 subunit of PtdIns 3-kinase was determined. Using anti-phosphotyrosine antibodies, p85 could be immunoprecipitated from lysates of PDGF-stimulated AG1523 cells, and to a lesser extent from NIH 3T3 fibroblasts expressing a constitutively active $p60^{c\text{-}src}$ kinase (results not shown), but not from resting AG1523 cells. {Phosphorylation of Tyr-527 suppresses the tyrosine kinase activity of $p60^{c\text{-}src}$. The Y527F mutation results, therefore, in increased levels of tyrosine-phosphorylated proteins in NIH $3T3^{c\text{-}srcF527}$ cells (for details see Amrein and Sefton [22]).} PDGF-mediated tyrosine phosphorylation of p85 was not affected by the presence of 1 $\mu$M wortmannin (Figure 2). Phosphorylation of PtdIns 3-kinase in the presence of wortmannin reports in intact PDGF-$\beta$ receptor autophosphorylation and correct interaction with p85.

Hypothetically, wortmannin might also cause the dissociation of the p85/p110 complex of PtdIns 3-kinase. In this case, p110 would be lost from immunoprecipitates, and in cells p110 would no longer be shuttled to the PDGF receptor. To test this possibility, anti-p85 immunoprecipitates were incubated with 1 $\mu$M wortmannin, which is 10 times the concentration used for the complete inhibition of PtdIns 3-kinase (Figure 2). Under these conditions, p110 was still detected by immunoblotting (Figure 3). Because both p85 and p110 are present in wortmannin-treated anti-p85 immunoprecipitates, but PtdIns 3-kinase is inactive, it is likely that wortmannin binds to the catalytic subunit p110. Preliminary experiments showed that high concentrations of ATP present during incubations with wortmannin can partly prevent the inhibition of the enzyme (M. P. Wymann and A. Arcaro, unpublished work). This suggests that the binding sites of wortmannin and ATP might lie in close proximity on p110.

Together with the inhibitory action of wortmannin on *in vitro* PtdIns3*P* and cellular PtdIns(3,4,5)$P_3$ production, these results illustrate that wortmannin blocks specifically the catalytic activity of PtdIns 3-kinase without affecting upstream signalling events.

This is in agreement with results obtained in neutrophils, where wortmannin inhibits formyl-methionyl-leucyl-phenylalanine (fMLF)-induced PtdIns(3,4,5)$P_3$ formation without affecting the PtdIns 4- and 5-kinases supplying PtdIns 3-kinase with its substrate, PtdIns(4,5)$P_2$ [21]. It has also been shown that micromolar concentrations of wortmannin do not disturb the fMLF-induced release of $Ca^{2+}$ from internal stores [27], which depends on the functioning of heterotrimeric G-proteins and phospholipase C producing Ins(1,4,5)$P_3$. Wortmannin has been shown to affect cell responses such as the respiratory burst [27,28] and phospholipase D activation [29,30] in neutrophils, and one type of calcium channel [31] and phosphorylation of pleckstrin [32] in platelets. Since the direct target of wortmannin was not identified until recently, the effects of the inhibitor should be re-evaluated considering the results presented here and in [21]. Wortmannin at micromolar concentrations can inhibit myosin light chain kinase, but does not affect the activities of protein kinase C, cyclic AMP-dependent kinase, calmodulin-dependent kinase II or cyclic GMP-dependent kinase under these conditions [33]. Therefore, and considering the low wortmannin concentrations shown here to inhibit PtdIns 3-kinase ($IC_{50}$ ~ 1 nM *in vitro*), this substance can be used as a specific inhibitor of PtdIns 3-kinase and might help to reveal the importance of PtdIns(3,4,5)$P_3$ signalling in response to surface receptor ligands such as PDGF, epidermal growth factor, insulin, the chemotactic peptide fMLF and others.

Most of the filamentous actin in resting fibroblasts is integrated in the actomyosin system of stress fibres. When stimulated with PDGF-BB, however, the cytoskeleton of these cells is reorganized and they display prominent circular membrane ruffles, which were suggested to be related to cell motility and chemotaxis [8].

As shown in Figure 4, such PDGF-stimulated actin rearrangements were completely abolished by 50–100 nM wortmannin. The fact that wortmannin-treated PDGF-stimulated cells maintained the appearance of their resting counterparts suggests a role for PtdIns 3-kinase in the signalling pathway mediating changes in the fibroblast cytoskeleton.

This view is in agreement with the finding that the cytosolic tyrosine kinase moiety (in particular Tyr-751 and Tyr-740) of the PDGF receptor is important for membrane ruffling and chemotactic responses of fibroblasts [12,34]. When phosphorylated, the latter tyrosine residues mediate the receptor interaction with PtdIns 3-kinase [4–6,9,10,35], and their replacement by phenylalanine abolishes chemotaxis and actin rearrangements in response to PDGF [12]. Still, one could argue that the effect of the Tyr-751 and Tyr-740 mutations is not mediated through the abolition of PtdIns 3-kinase translocation and activation itself, but through the elimination of a possible shuttle function

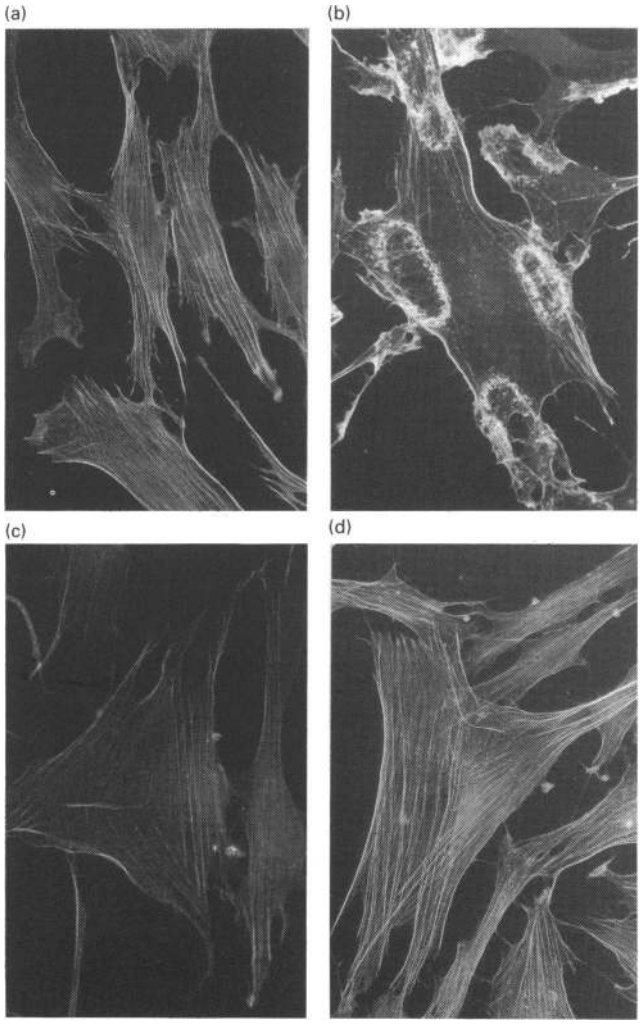


**Figure 4 Inhibition of PDGF-mediated actin rearrangements by wortmannin**

Serum-starved, subconfluent AG1523 cells were kept for 30 min at 37 °C in the absence (**a**, **b**) or presence of wortmannin (50 nM for **c**; 100 nM for **d**). Vehicle (**a**) or PDGF (30 ng/ml; **b**–**d**) was added for 10 min at 37 °C, before cells were fixed, permeabilized, stained for filamentous actin with fluorescein–phalloidin and mounted for fluorescence microscopy.

provided by PtdIns 3-kinase for associated proteins. Combined with the data presented here, however, the results provide convincing evidence for PtdIns(3,4,5)$P_3$ being a necessary signal for actin reorganization, and possibly for motility and chemotaxis in fibroblasts.

In contrast to the situation in fibroblasts, pseudopod formation in neutrophils is only modulated (wortmannin-treated neutrophils respond to fMLF with a series of actin polymerization/depolymerization cycles [25]), but not abolished, when PtdIns 3-kinase is inhibited [21]. The reason for the different behaviour of the two cell types is not yet clear, but might emerge from the different cytoskeletal structures and actin isoforms present in fibroblasts and neutrophils.

Because of their prominent and easily detectable responses, fibroblasts might provide an excellent model system with which to investigate the actions of PtdIns(3,4,5)$P_3$ on the cytoskeleton and to identify the actin-binding proteins involved.

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