RESEARCH COMMUNICATION

A role for the actin cytoskeleton in the hormonal and growth-factor-mediated activation of protein kinase B

Karine PEYROLLIER^{*1}, Eric HAJDUCH^{*1}, Alexander GRAY[†], Gary J. LITHERLAND^{*}, Alan R. PRESCOTT^{*}, Nick R. LESLIE^{†1} and Harinder S. HUNDAL^{*2}

^{*}School of Life Sciences and the Division of Signal Transduction Therapy[†], Medical Sciences Institute/Wellcome Trust Biocentre Complex, Dow Street, University of Dundee, Dundee DD1 5EH, U.K.

We show here that cytochalasin D-induced depolymerization of actin filaments markedly reduces the stimulus-dependent activation of protein kinase B (PKB) in four different cell types (HEK-293 cells, L6 myotubes, 3T3-L1 adipocytes and U87MG cells). HEK-293 cells expressing the pleckstrin homology (PH) domains of PKB and general receptor for phosphoinositides-1 (GRP1) fused to green fluorescent protein (GFP) were used to monitor production of 3-phosphoinositides in the plasma membrane. Disassembly of the actin cytoskeleton significantly reduced the insulin-mediated translocation of both PKB-PH-GFP and GRP1-PH-GFP to the plasma membrane, consistent with diminished synthesis of 3-phosphoinositides. Actin depolymerization did not affect the hormonal activation of phosphoinositide 3-kinase (PI 3-kinase), and since cytochalasin D treatment also led to reduced platelet-derived growth factor

(PDGF)-induced phosphorylation of PKB in U87MG cells, a PTEN (phosphatase and tensin homologue deleted on chromosome 10) null cell line, lipid phosphatase activity was unlikely to account for any reduction in cellular 3-phosphoinositides. Withdrawal of cytochalasin D from the extracellular medium induced actin filament repolymerization, and reinstated both the recruitment of PH-GFP fusion proteins to the plasma membrane and PKB activation in response to insulin and PDGF. Our findings indicate that an intact actin network is a crucial requirement for PI 3-kinase-mediated production of 3-phosphoinositides and, therefore, for the activation of PKB.

Key words: glycogen synthase kinase-3, insulin, phosphatase and tensin homologue deleted on chromosome 10, phosphoinositide 3-kinase, platelet-derived growth factor.

INTRODUCTION

The serine/threonine kinase protein kinase B (PKB), which lies downstream of phosphoinositide 3-kinase (PI 3-kinase), plays a key role in regulating diverse cellular functions in response to signalling initiated by activated hormone and growth factor receptors (for review see [1]). PKB activation depends upon its phosphorylation of threonine-308, located within the kinase activation loop, and serine-473, which lies in the C-terminal domain of the kinase [2]. PKB phosphorylation is preceded by its translocation to the plasma membrane [3], an event which involves the binding of 3-phosphoinositides [PtdIns(3,4)P₂ or PtdIns(3,4,5)P₃] to the N-terminal pleckstrin homology (PH) domain of PKB [4,5]. The binding of phosphoinositides to the PH-PKB domain is considered to be important in inducing conformational changes which enable the threonine-308 site to be phosphorylated by an upstream kinase called 3-phosphoinositide-dependent kinase 1 (PDK1) [6,7], and for the serine-473 site to be phosphorylated by an as yet unidentified kinase termed PDK2 [1]. Activated PKB is then able to directly phosphorylate and modulate the activity of downstream target proteins, such as glycogen synthase kinase-3 (GSK-3) [8] and FKHR, a member of the 'forkhead' family of transcription factors [9], as well as influence multiple cell functions such as differentiation, glucose metabolism and cell survival [10].

While the recruitment of PKB to the plasma membrane is essential for its subsequent activation, very little is known about

the mechanics of the translocation event, or whether there is a requirement for other cellular components to facilitate this process. There is accumulating evidence showing that molecules involved in proximal insulin signalling, such as insulin receptor substrate-1 and PI 3-kinase, can associate with the membrane cytoskeleton [11], and that disassembly of the actin network, for example, suppresses the activation of key cell responses to insulin, such as glucose transport [12]. Since insulin and growth factors promote the reorganization of actin filaments, this process may require the association of early signalling elements with the cytoskeleton, and could serve to co-localize activated receptor complexes with molecules involved in signalling to diverse end points. In the present study we have investigated the role played by the actin cytoskeleton in the hormonal- and growth-factor-mediated activation of PKB, and show for the first time that depolymerization of the actin cytoskeleton in four different cell types results in a significant loss in the activation of PKB. This impaired activation of PKB stems from a loss in the stimulus-dependent production of 3-phosphoinositides, and can be reversed upon repolymerization of the actin network.

EXPERIMENTAL

Materials

α -Minimal essential medium (α -MEM), Dulbecco's modified Eagle's medium, fetal calf serum, antimycotic/antibiotic solution

Abbreviations used: PKB, protein kinase B; PH domain, pleckstrin homology domain; GRP1, general receptor for phosphoinositides-1; GFP, green fluorescent protein; PI 3-kinase, phosphoinositide 3-kinase; PDGF, platelet-derived growth factor; PDK1, 3-phosphoinositide-dependent kinase 1; GSK-3, glycogen synthase kinase-3; α -MEM, α -minimal essential medium; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

[†] These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail h.s.hundal@dundee.ac.uk).

were from Life Technologies. All other reagent-grade chemicals were obtained from Sigma–Aldrich. Antibodies against PKB α and PKB β and peptide substrates for kinase assays were generously provided by Professor Sir Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee). Phospho-specific antibodies to PKB and GSK-3 were from New England Biolabs (Hitchin, Herts., U.K.). An antibody against the p85 subunit of PI 3-kinase was purchased from UBI (Lake Placid, NY, U.S.A.). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from Scottish Antibody Production Unit (Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.). Protein A- and Protein G–Sephacrose beads were purchased from ICN Biomedicals.

Cell culture

HEK-293 cells and U87MG cells were maintained in Dulbecco's modified Eagle's medium and α -MEM respectively and cultured as described previously [13,14]. L6 muscle cells were cultured to the stage of myotubes in α -MEM containing 2% (v/v) fetal calf serum, as reported previously [15], and 3T3-L1 cells (provided by Dr Howard Green, Harvard University) were differentiated into adipocytes, as described previously [16].

SDS/PAGE and immunoblotting

Cell lysates (50 μ g) from L6 myotubes, HEK-293 cells, 3T3-L1 adipocytes and U87MG cells were subjected to SDS/PAGE on 10% resolving gels and transferred on to nitrocellulose membranes, as reported previously [15]. Nitrocellulose membranes were then probed with anti-phospho-antibodies against PKB serine-473 and GSK-3, or antibodies recognizing native PKB α and GSK-3 β (all at 1:1000 dilution). Primary antibody detection was performed using horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution), and visualized using enhanced chemiluminescence on Konica Medical film (Hohenbrunn, Germany).

PKB activities in L6, 3T3-L1 and HEK-293 cell lysates

L6 myotubes, HEK-293 cells and 3T3-L1 adipocytes were exposed to 2 μ M cytochalasin D for 2 h and to 100 nM insulin for 10 min. Cells were then lysed using lysis buffer [50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM Na₄P₂O₇, 1 μ M microcystin-LR, 270 mM sucrose, 1 mM benzamide, 10 μ g/ml leupeptin and 0.1% (v/v) 2-mercaptoethanol]. PKB α and PKB β were immunoprecipitated from lysates using antibodies against their respective C-terminal domains, and kinase activities assayed using the synthetic peptide substrate Crosstide as described previously [8]. Protein concentrations were determined using the method of Bradford [17].

PDK1 activity

PDK1 was immunoprecipitated from L6 lysates using a PDK1-specific antibody [18]. The immunoprecipitates were washed twice with lysis buffer containing 0.5 M NaCl and twice with a buffer containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Immunoprecipitated PDK1 was used to phosphorylate a synthetic peptide called 'PDKtide' as described previously [19].

Assay of PI 3-kinase activity in L6 lysates

L6 myotubes were treated with insulin and/or cytochalasin D and lysed as described above. Lysates were incubated with anti-

p85 antibody complexed to Protein G–Sephacrose beads. Immuno-precipitates were washed and incubated with phosphatidyl-inositol (0.1 mg/ml) for 20 min at 30 °C, in a buffer containing 50 mM [γ -³²P]ATP, 1.2 mM Na₃VO₄, 5 mM MgCl₂ and 25 mM Hepes (pH 7.4), and products separated by TLC on silica gel G-60, as described previously [20]. ³²P-labelled PI3P spots were visualized on Konica Medical film.

Cell transfection and immunofluorescence microscopy

HEK-293 cells were grown on glass coverslips in 6-well plates, and transfected with the green fluorescent protein (GFP)-tagged N-terminal pleckstrin homology domain (PH domain) of PKB and GFP-tagged PH domain of mouse general receptor for phosphoinositides-1 (GRP1), as described previously [13]. Cells were incubated in serum-free Dulbecco's modified Eagle's medium 24 h after transfection for 3 h and transferred into Hepes-buffered saline [20 mM sodium Hepes (pH 7.4), 140 mM NaCl, 2.5 mM MgSO₄, 5 mM KCl and 1 mM CaCl₂] 15 min prior to any additions. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature, before being washed 3 times in PBS and mounted on to slides for confocal microscopy. For some experiments, HEK-293 cells and L6 myotubes were grown on glass coverslips, deprived of serum and incubated in the absence or presence of 2 μ M cytochalasin D, or allowed to recover in normal growth media having already been incubated with cytochalasin D for 2 h. Cells were then fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 and subsequently incubated with rhodamine-labelled phalloidin for 1 h. Cells were washed three times with PBS prior to mounting on to slides for confocal microscopy.

Statistical analysis

Statistical analysis was carried out using a two-tailed Student's *t* test. Data were considered statistically significant at *P* values $\leq$ 0.05.

RESULTS AND DISCUSSION

To assess the importance of the actin cytoskeleton for insulin signalling we first monitored the effects of incubating HEK-293 cells and L6 myotubes with 2 μ M cytochalasin D for 2 h. This treatment resulted in the depolymerization of actin filaments and the aggregation of actin in both cell types, as visualized using rhodamine-labelled phalloidin and confocal microscopy. The disruption seen in the actin network in both cell types was reversed upon washing cells free of cytochalasin D and allowing them to recover in media lacking cytochalasin D for a period of 2 h (Figure 1).

We next assessed whether the actin cytoskeleton participated in the activation of PKB by insulin. Probing cell lysates with phospho-specific PKB antibodies revealed that insulin promoted phosphorylation of PKB serine-473 in both L6 myotubes and HEK-293 cells (Figure 2a). This phosphorylation was virtually undetectable in L6 myotubes and reduced by more than 60% in HEK-293 cells by prior incubation of cells with cytochalasin D for 2 h (Figures 2a). A diminished insulin-stimulated phosphorylation of PKB serine-473 was also observed in 3T3-L1 adipocytes following preincubation with cytochalasin D (Figure 2a), and analysis of threonine-308 PKB phosphorylation yielded identical results in all three cell types (Figure 2a). Interestingly, PKB phosphorylation was reinstated fully when cytochalasin D was withdrawn from the medium and cells allowed to recover for 2 h prior to restimulation with insulin. To substantiate these findings we immunoprecipitated PKB α from L6 myotubes and HEK-293

Figure 1 Disruption of actin network by cytochalasin D treatment in HEK-293 cells and L6 myotubes

Serum-deprived HEK-293 cells and L6 myotubes were incubated in the absence or presence of 2 μ M cytochalasin D (CD) for 2 h. Cells were subsequently processed for visualizing the actin network by confocal microscopy, as described in the Experimental section, or washed three times with appropriate culture media lacking cytochalasin D, and then allowed to recover in culture media for 2 h prior to labelling the actin network with rhodamine-labelled phalloidin and confocal microscopy.

Figure 2 Effects of cytochalasin D and latrunculin B (LB) on insulin-induced phosphorylation of PKB and GSK-3 and PI 3-kinase activity in L6 myotubes, HEK-293 cells and 3T3-L1 adipocytes

Serum-deprived L6 myotubes, HEK-293 cells and 3T3-L1 adipocytes were incubated in the absence or presence of 2 μ M cytochalasin D (CD) or 2 μ M latrunculin B (LB) for 2 h. In some experiments cells were washed (w) with culture media following treatment with cytochalasin D and allowed to recover for 2 h in media lacking cytochalasin D. Following treatment with cytochalasin D or recovery, cells were incubated with 100 nM insulin (I) for 10 min. Lysates from cells not subjected to treatment with cytochalasin D or insulin were used as control (C). (a, b) Cells were lysed and immunoblotted using phospho-specific antibodies against PKB serine-473, threonine-308 and native PKB antibody or (c) anti-phospho-specific antibodies to GSK-3 α and GSK-3 β and native GSK-3 β (data shown in panels b and c are from experiments with L6 myotubes). (d) L6 myotubes were lysed and PI 3-kinase activity assessed in p85 immunoprecipitates as described in the Experimental section. The immunoblots shown are representative of at least three independent experiments.

Table 1 Effects of insulin (100 nM) and cytochalasin D (CD; 2 μ M) treatment on PKB and PDK1 activity

Cells were incubated with insulin for 10 min or exposed to the hormone after a 2 h incubation with CD or a 2 h recovery period that followed withdrawal of CD from the extracellular medium. PKB and PDK1 were immunoprecipitated and activity assessed as described in the Experimental section. Values represent fold-change relative to the unstimulated condition. Data are means $\pm$ S.E.M. from three separate experiments. Asterisks indicate significant differences ($P < 0.05$) from the unstimulated value, and daggers indicate significant differences ($P < 0.05$) from the value obtained with insulin treatment alone.

	L6 myotubes PKB α	3T3-L1 adipocytes PKB β	HEK 293 cells PKB α	L6 myotubes PDK1
Unstimulated	1.0	1.0	1.0	1.0
Insulin	14.6 $\pm$ 0.9*	17.7 $\pm$ 1.2*	2.4 $\pm$ 0.3*	0.91 $\pm$ 0.1
CD/insulin	3.6 $\pm$ 1.3†	9.9 $\pm$ 2.6†	1.3 $\pm$ 0.2†	1.3 $\pm$ 0.3
CD/wash/insulin	16.3 $\pm$ 2.6*	17.0 $\pm$ 3.5*	2.6 $\pm$ 0.6*	1.0 $\pm$ 0.1

cells, and PKB β from 3T3-L1 adipocytes (these isoforms were studied as they are the predominant forms activated by insulin in these cell types [21]) following treatment with cytochalasin D and insulin, and assayed the activity of the kinase. In line with the immunoblot data, Table 1 shows that insulin activated PKB significantly in all three cell types, and this was reduced significantly and reversibly by prior cytochalasin D treatment. Use of a different actin-disrupting agent, latrunculin B, which destabilizes actin filaments and promotes the sequestration of actin monomers yielded similar results (Figure 2b).

The activation of PKB by insulin and growth factors results in the phosphorylation and inactivation of its downstream target, GSK-3 [8]. We therefore investigated whether insulin-induced GSK-3 phosphorylation was also modulated by cyto-

chalasin D treatment. Consistent with the loss in PKB activation, Figure 2(c) shows that the insulin-induced phosphorylation of both GSK-3 α and GSK-3 β did not take place following incubation of L6 cells with cytochalasin D, but could be reinstated when cells were allowed to recover subsequently in culture medium that did not contain cytochalasin D.

The hormonal- and growth-factor-mediated activation of PKB is PI 3-kinase-dependent [1], and PI 3-kinase has been reported to associate with detergent-insoluble structures that include cytoskeletal elements [11,22]. Furthermore, it has been suggested that actin filaments may play an important role in localizing PI 3-kinase appropriately to facilitate downstream signal propagation [11,22]. To address whether the impaired activation of PKB in cells treated with cytochalasin D stems from a loss in PI 3-kinase activation, we immunoprecipitated the p85 subunit of PI 3-kinase and assayed kinase activity *in vitro*. Incubating L6 cells with insulin caused a near 3-fold increase in PI 3-kinase activity that was not suppressed by preincubation of cells with cytochalasin D (Figure 2d), a finding consistent with previous work [22,23]. Thus while disruption of the actin network in L6 cells substantially reduces the hormonal activation of PKB (Figure 2a and Table 1), it has little impact on the catalytic activation of PI 3-kinase.

The phosphorylation of PKB threonine-308 is mediated by PDK1 and it is possible that reduced phosphorylation of this site in cells treated with cytochalasin D stems from impaired PDK1 activity. However, analysis of PDK1 activity revealed that disruption of the actin cytoskeleton in L6 cells did not affect the catalytic activity of this kinase when assayed *in vitro* (Table 1). Since PDK1 is known to be constitutively active as a result of binding the low levels of PtdIns(3,4) P_2 or PtdIns(3,4,5) P_3 which are present in the plasma membrane of unstimulated cells [24], our findings imply that disruption of the actin cytoskeleton has

Figure 3 Effects of cytochalasin D and insulin on the localization PH-PKB-GFP and PH-GRP1-GFP fusion proteins in HEK-293 cells

(a, f) Unstimulated cells; (b, g) cells incubated with 2 μ M cytochalasin D alone; (c, h) cells stimulated for 10 min with 100 nM insulin; (d, i) cells pretreated with 2 μ M cytochalasin D for 2 h, then stimulated with 100 nM insulin for 10 min; (e, j) cells pretreated with 2 μ M cytochalasin D for 2 h, washed and allowed to recover in culture media lacking cytochalasin D for 2 h prior to stimulation with 100 nM insulin for 10 min. Arrows indicate sites of surface localization of GFP fusion proteins. The confocal micrographs are representative of four independent experiments. Scale bar = 10 μ m.

little impact on the basal level of these lipids. Insulin fails to activate PDK1 (Table 1), consistent with the view that the stimulus-mediated increase in $\text{PtdIns}(3,4)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$ does not activate PDK1, but serves primarily to co-localize the kinase and its substrates. Phosphorylation of the two regulatory sites on PKB is preceded by recruitment of the kinase to the cell surface, and this requires an insulin- and growth-factor-mediated increase in cellular $\text{PtdIns}(3,4)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$ in the cell membrane [1]. It is thus plausible that while insulin-stimulated PI 3-kinase activity was not affected in cells lacking an intact actin cytoskeleton, its ability to phosphorylate phosphoinositides may be impaired *in vivo*. To address whether disassembly of the actin cytoskeleton inhibits the insulin-dependent synthesis of 3-phosphoinositides, we transfected HEK-293 cells with the PH domains of both PKB and GRP1 fused to GFP. Both fusion proteins have been shown to act as sensitive and selective probes for detecting the generation of $\text{PtdIns}(3,4)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$, and their translocation to the plasma membrane in response to stimuli such as insulin correlates very tightly with the cellular production of these 3-phosphoinositides [13]. In unstimulated HEK-293 cells both GFP-fusion proteins were predominantly localized in the cytosolic compartment, though some constitutive surface localization of PH-PKB-GFP and PH-GRP1-GFP was detectable (indicated by arrows in Figure 3a and 3f), which was not affected by treatment of cells with cytochalasin D (Figures 3b and 3g). The constitutive surface localization may be a consequence of overexpression of these fusion proteins, altering the natural ratio of surface-to-cytosolic localization. Alternatively, the observations may merely reflect a small minority of endogenous surface localization of PH domain-containing proteins in the basal state, visible only due to overexpression of the GFP moiety. Stimulation with insulin induced a striking redistribution of both PH-PKB-GFP and PH-GRP1-GFP from the cytosolic compartment to the cell periphery (Figures 3c and 3h), which was suppressed markedly in cells that had been preincubated with cytochalasin D (Figures 3d and 3i). Nevertheless, some plasma membrane labelling of these GFP fusion proteins persists in cells treated with cytochalasin D (indicated by arrows in Figures 3d and 3i), possibly reflecting incomplete depolymerization of actin filaments in these cells, which may support limited synthesis of 3-phosphoinositides and account for the partial activation of PKB (Figure 2a and Table 1). Allowing the actin network to reform restored the ability of insulin to induce PH domain translocation to the plasma membrane (Figures 3e and 3j). Since translocation of the PH domains of both PKB and GRP1 correlates closely with $\text{PtdIns}(3,4,5)\text{P}_3$ production in HEK-293 cells [13], our data would indicate that an intact actin cytoskeleton is crucial for enabling an insulin-stimulated increase in 3-phosphoinositides. Given that we did not directly monitor the production of 3-phosphoinositides *in vivo*, we cannot exclude the possibility that the impaired translocation of our GFP-PH fusion proteins reflect defects in the plasma membrane recruitment of the native PKB and GRP1 proteins, unrelated to changes in cellular 3-phosphoinositides. However, we feel it is unlikely that the translocation of isolated PH domains, which represent 3-phosphoinositide binding polypeptide motifs, would be affected by circumstances other than the generation of these lipids.

It was possible that the diminished cell surface PH domain recruitment seen following disruption of the actin network could reflect the increased dephosphorylation of 3-phosphoinositides by a specific lipid phosphatase, such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which has been shown to antagonise PI 3-kinase and PKB signalling [25,26]. To address whether PTEN participates in the impaired activation of

Figure 4 Effects of cytochalasin D on PDGF-induced phosphorylation of PKB in U87MG cells

U87MG cells were incubated in MEM medium containing 1% fetal bovine serum for 5 h. Cells were subsequently incubated with PDGF (5 $\mu\text{g}/\text{ml}$) for 10 min or exposed to PDGF following incubation with 2 μM cytochalasin D (CD) for 2 h. Cells were lysed and lysates immunoblotted using phospho-specific antibodies against PKB serine-473, threonine-308 or an antibody to native PKB. Lysates from cells not subjected to treatment with CD or PDGF were used as control (C). The top panel shows the effects of cytochalasin D treatment alone on PKB threonine-308 phosphorylation. Blots in the lower panel show data from two separate experiments run side by side.

PKB seen in cytochalasin D-treated cells, we used a glioblastoma-derived PTEN null cell line U87MG [14]. These cells display constitutively high levels of $\text{PtdIns}(3,4,5)\text{P}_3$ and an elevated basal PKB activity, which could be lowered by incubating cells for 4 h in medium containing low serum. We detected weak phosphorylation of PKB serine-473 and threonine-308 in lysates prepared from unstimulated U87MG cells, which was not significantly affected by treatment of cells with cytochalasin D alone, but which could nevertheless be stimulated noticeably by platelet-derived growth factor (PDGF) (Figure 4), as could PKB activity (results not shown). As with the other cell types studied, these effects are not observed when U87MG cells were pre-incubated with cytochalasin D prior to stimulation with PDGF (Figure 4). This suggests that the impaired hormonal or growth factor-mediated activation of PKB is more likely to be explained by reduced synthesis of $\text{PtdIns}(3,4)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$ than by increased dephosphorylation of these lipids.

How the actin network is involved in propagating hormone and growth factor signalling is unclear at present, but our data is consistent with the suggestion that actin filaments may serve to localize activated PI 3-kinase molecules in close proximity to their substrates in the plasma membrane. Both PI 3-kinase and $\text{PtdIns}(4,5)\text{P}_2$, a PI 3-kinase substrate, localize to actin-rich structures [27]. Since the cytoskeleton is closely associated with the inner leaflet of the plasma membrane, it is plausible that association of PI 3-kinase with the actin cytoskeleton serves to generate $\text{PtdIns}(3,4,5)\text{P}_3$ from phosphoinositide $\text{PtdIns}(4,5)\text{P}_2$, thereby enabling recruitment of signalling molecules such as PKB to the plasma membrane. The precise mechanisms that

govern the stimulus-induced association of PI 3-kinase with the actin cytoskeleton remain unknown, but understanding their nature clearly has important implications for downstream signalling, and hence for the control of diverse and vital cell functions.

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