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Research Article

Targeting of the Akt/PKB kinase to the actin skeleton

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Abstract. Serine/threonine kinase Akt/PKB intracellular distribution undergoes rapid changes in response to agonists such as Platelet-derived growth factor (PDGF) or Insulin-like growth factor (IGF). The concept has recently emerged that Akt subcellular movements are facilitated by interaction with nonsubstrate ligands. Here we show that Akt is bound to the actin skeleton in in situ cytoskeletal matrix preparations from PDGF-treated Saos2 cells, suggesting an interaction between the two proteins. Indeed, by immunoprecipitation and subcellular fractioning, we demonstrate that endogenous Akt and actin physically interact. Using recombinant proteins in in vitro binding and overlay assays, we further demonstrate that Akt interacts with actin directly. Expression of Akt mutants strongly indicates that the N-terminal PH domain of Akt mediates this interaction. More important, we show that the partition between actin bound and unbound Akt is not constant, but is modulated by growth factor stimulation. In fact, PDGF treatment of serum-starved cells triggers an increase in the amount of Akt associated with the actin skeleton, concomitant with an increase in Akt phosphorylation. Conversely, expression of an Akt mutant in which both Ser473 and Thr308 have been mutated to alanine completely abrogates PDGF-induced binding. The small GTPases Rac1 and Cdc42 seem to facilitate actin binding, possibly increasing Akt phosphorylation.

Key words. Akt; cytoskeleton; PDGF; Rac; Cdc42; GFP-actin.

Akt (also called PKB) is a serine/threonine kinase involved in promotion of cell survival, proliferation and metabolic responses [1–3], downstream of the phosphoinositide 3-kinase (PI 3-kinase) signaling pathway [4]. Akt activation is a multistep process involving coordinated actions of several catalytic and noncatalytic molecules. An early and obligatory step is Akt translocation from the cytosol to the plasma membrane. Akt is a predominantly cytosolic enzyme in resting cells; however, generation of PI 3-kinase lipid products recruits Akt to the plasma membrane through its N-terminal PH domain [5, 6], resulting in a conformational change which induces phosphorylation at two residues, Thr308 and Ser473, of the membrane-bound protein. Thr308 lies in the activation loop of the kinase domain and is phosphorylated by the phosphoinositide-dependent kinase PDK1, while Ser473 is located in a noncatalytic region termed the hydrophobic motif and is phosphorylated by an upstream kinase, provisionally termed PDK2, whose identity is still being discussed [7–9]. Recent evidence suggests that phosphorylation at Thr308 depends on prior localization to the plasma membrane and phosphorylation at Ser473 [10]. The activated protein is then redistributed to cytoplasm and nucleus [11], where it phosphorylates substrates whose phospho-acceptor sites are

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specified by the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa and Zaa are small residues and Hyd is a hydrophobic residue [3]. One of the first identified substrates of Akt was glycogen synthase kinase 3 (GSK3). Phosphorylation of GSK3 by Akt results in its inactivation, and is therefore a device to control glucose utilization and activation of glycogen synthesis [3]. Akt can also promote cell survival by inhibiting proteins that mediate apoptosis. An Akt substrate that has a key role in this mechanism is the pro-apoptotic Bcl2 family member BAD. Following phosphorylation by Akt, BAD is sequestered by 14-3-3 proteins, a condition which prevents its interaction with Bcl-XL, thereby suppressing apoptosis [12]. Moreover, in the nucleus Akt can phosphorylate the FOXO members of the Forkhead family, leading to nuclear export and inactivation of these transcription factors. Regulation of FOXO transcriptional activity has been implicated in cell cycle and cell death control by Akt [12]. Akt has also been shown to phosphorylate the cyclin-CDK inhibitor p21. Phosphorylated p21 is seized into the cytoplasm by 14-3-3 proteins, where it cannot carry out its function [12, 13].

It has been demonstrated that Akt not only interacts with substrate proteins, but can also form complexes with proteins that behave as regulators rather than substrates of Akt [14]. Interaction with nonsubstrate proteins can affect Akt kinase activity, as in the case of TCL1, CTMP, Hsp90/Cdc37, Hsp27 and Btk [14, 15], or regulate Akt intracellular localization, as in the case of TCL1 [16, 17], keratin 10, Grb10 [14] and periplakin [18], possibly allowing the activation of a localized signaling network by targeting Akt to its location.

The data presented here indicate that Akt can interact with actin directly, both in vitro and in vivo. More important, we show that the partition between actin-bound and -unbound Akt is not constant, but is modulated by PDGF and IGF I. Akt phosphorylation seems to be a requisite for Akt to associate with actin. These and other observations suggest that Akt is targeted to actin in response to precise signals, and may help to explain how Akt is redistributed inside the cell following activation.

Materials and methods

Antibodies and reagents

The following antibodies were used: goat polyclonal anti-Akt and goat polyclonal anti-actin (Santa Cruz Biotechnology, CA); mouse monoclonal anti-HA 12CA5 (Roche, Germany); rabbit polyclonal anti-Akt and anti-phosphoAkt (S473) (Cell Signaling Technologies, New England Biolabs, UK); mouse monoclonal anti-Cdc42 and anti-Rac1 (Transduction Lab, Becton Dickinson, Biosciences, CALIF.), Horseradish peroxidase-conjugated (HRP) secondary antibodies as well as chemiluminescence (ECL) reagents were from Amersham-Pharmacia Biotech, while secondary antibodies for immunofluorescence were from Jackson ImmunoResearch Laboratories (West Grove PA). The protease inhibitor cocktail was from Sigma.

All electrophoresis reagents, nitrocellulose and protein assay kits were from Bio-Rad. Thrombin, Isopropyl beta-D-1thiogalactopyranoside and lysozyme were from Sigma. FuGENE 6 transfection reagent was from Roche (Germany). The G-actin/F-actin fractioning kit was from Cytoskeleton (Denver, CO). All other chemicals were of the highest quality available.

Expression Plasmids

pEGFP-C1was purchased from Clontech Laboratories. EGFP-HA-Akt wild type (wt) and EGFP-HA-AH-Akt (encoding the PH domain) were obtained by polymerase chain reaction (PCR)-cloning of the murine HA-Aktα complementary DNA (cDNA) into the EcoRI site of pEGFP-C1 in frame with the green fluorescent protein (GFP) start codon [19]. The Δ1-147HA-Akt mutant, lacking the entire PH domain, was cloned in pcDNA3. GST-Akt and GST-HA-AH-Akt, HA-Akt Δ-PH (11–60) were a kind gift of Alex Toker (Department of Pathology, Harvard Medical School, Boston). Wild-type AU5-tagged Cdc42 (Cdc42 wt) and AU5-tagged Rac1 (Rac1 wt) were a kind gift of Keith Burridge (UNC Chapel Hill), as were dominant-negative Myc-tagged N17-Cdc42 (Cdc42N17) and dominant-negative Myc-tagged Rac1N17.

Cell culture and transfections

Human osteosarcoma Saos2 cells (ATCC, 44° P) and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium additioned with 10% heat-inactivated foetal bovine serum (FBS, EuroClone, UK). Cells were kept at 37 °C in a 5% CO_2 humidified atmosphere. For immunofluorescence experiments, Saos2 cells seeded on glass coverslips the day before were transfected using the FuGENE 6 reagent (Roche). Typically, 2×10^5 cells per 35-mm dish were transfected with 1 µg of each DNA construct, combined with empty vector for a total of 2 µg per well. The DNA/FuGENE 6 mix (3:1 liposome:DNA ratio) was overlayed onto cells in medium without serum and left for the duration of the experiment, following the manufacturer's instructions. Where indicated, 293 cells were used due to higher transfection efficiency. Transient transfections of 293 cells were performed by the calcium phosphate method, as described previously [20]. Transfected cells used for these experiments contained levels of Akt no greater than two- to threefold endogenous levels. After transfection, cells were allowed to express the protein for 24 h, then were washed in PBS and scraped directly into lysis buffer.

Preparation of cell extracts

Whole cell lysates were obtained from subconfluent cells, extracted with radio immuno protection assay (RIPA) buffer (1 ml/60-mm Petri dish) (50 mM Tris-Cl, pH 7.8, containing 1% Nonidet P40, 140 mM NaCl, 0.1% SDS, 0.1% Na deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 10 mM *o*-nitrophenylphosphate and freshly added 1× protease inhibitor cocktail), directly into the plate for 5 min at 4°C. Lysates were then cleared by centrifugation for 15 min in a refrigerated microfuge, max speed, and used for pull-down or immunoprecipitation experiments, as described below. 50 µl of the total lysate were immediately boiled in SDS sample buffer, resolved and revealed with specific antibodies, as noted. G- and F-actin fractioning was carried out with the Cytoskeleton kit BK037, following the manufacturer's instructions. Briefly, cells were homogenized in F-actin stabilization buffer prewarmed at room temperature (50 mM Pipes buffer pH 6.9 containing 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% v/v glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 1% Tween 20 and freshly added 1× protease inhibitor cocktail), at a ratio of 20 volumes of buffer per volume of cell pellet. G- and F-actin pools were then separated by centrifugation at 100,000 × g for 60 min. The F-actin-containing pellet was resuspended to the same volume of buffer as the G-actin-containing supernatant, and both were used for immunoprecipitation assay.

Immunoprecipitation

Lysates (1 mg) in RIPA buffer were precleared for 30 min at 4°C with 20 µl of protein A/G agarose slurry, centrifuged, and the supernatants were incubated for 4 h with anti-Akt (2 µg), anti-actin (3 µg) or anti-HA (1 µg), followed by 1 h incubation with 30 µl of protein A/G agarose slurry (Oncogene) at 4°C with shaking. Pellets were washed three times with the lysis buffer containing 450 mM NaCl, once with Tris-HCl, pH 7.5, and boiled in Laemmli sample buffer. Samples were resolved on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose, probed with specific primary antibodies, and HRP-conjugated second antibodies, and developed with ECL (Amersham-Pharmacia). The following dilutions were used for Western blotting analysis: anti-HA, anti-actin, anti-Rac1, anti-Akt and anti-Akt S473 1:1000; anti-Cdc42 1:250; HRP-conjugated secondary antibodies 1:3000.

In vitro binding

Purified rabbit skeletal muscle actin (2 µg) was incubated with 8 µg of the indicated, purified GST fusion proteins bound to glutathione (GSH)-sepharose beads for 1 h at 4°C, or GSH-sepharose beads, as noted. The beads were washed twice with RIPA buffer, once with Tris-HCl pH 7.5, and boiled in Laemmli sample buffer. Complexes were analysed in 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

Overlay Assay

Purified His_{6}-Akt (0.1, 0.5, 1 µg) or GST-AH-Akt (0.1, 0.2, 0.3 µg) was resolved on 7.5% polyacrylamide gels and blotted onto nitrocellulose membranes. After blocking with 3% (w/v) bovine serum albumine (Sigma) for 1 h at 4°C, membranes were renatured in binding buffer [300 mM KCl, 0.1% (v/v) Tween-20 and 20 mM Tris-HCl, pH 7.5], for 30 min at 4°C with gentle shaking. The control filter was left in renaturing buffer, while the second filter was incubated with purified actin (Sigma) in the same buffer, at a final concentration of 30 µg/ml (in molar excess as compared with Akt), at 4°C for 2 h with gentle stirring. After extensive washing to remove unbound protein, the two membranes were incubated with anti-actin and developed by ECL for the same time span.

Immunofluorescence and in situ matrix preparation

For immunofluorescence studies, Saos2 cells seeded on glass coverslips were transiently transfected as described above, then fixed with 2% (wt/vol) formaldehyde in PBS for 20 min at room temperature, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min and blocking with 4% BSA in PBS for 1 h before addition of anti-Akt (1:50) or anti-phosphoAkt (1:50) (IHC specific, Cell Signaling Tech., New England Biolabs) diluted in blocking buffer, for 1 h. After three washes in PBS, cells were incubated with either Cy3- or Fluorescein Isothio-cyanate (FITC)-conjugated secondary antibody for 45 min in blocking buffer. For actin staining, preparations were incubated with anti-actin (Sigma), diluted in blocking buffer, for 1 h. After washing in PBS, glass coverslips were incubated with secondary antibody (1:5000) for 30 min in blocking buffer and mounted on anti-fade solution (Molecular Probes). Colocalization between actin and Akt was calculated by LaserPix software (Bio-Rad) using the overlap coefficient, as previously described [21, 22].

For in situ matrix preparation, cells were seeded and transfected as above, then treated as described [23]. Briefly, cells were incubated for 10 min at room temperature with 10 mM Tris-Cl, pH 7.4, 5 mM MgCl2, 150 mM NaCl (Tris, Sodium, Magnesium buffer), 1% Nonidet P40 and freshly added protease inhibitors. After washings with TSM buffer, cells were treated with DNAse I (20 U/ml in TSM) at room temperature for 1 h. After two washes in ice-cold TSM buffer, cells were extracted twice with 2M NaCl and washed in PBS, then fixed in 2% formaldheyde at room temperature.

Confocal microscopy was performed using a laser scanner (Radiance 2000, Bio-Rad) attached to a Nikon microscope (Eclipse TE300).

Results

Akt colocalizes with actin in in situ cytoskeletal preparations

The intracellular localization of Akt has been described in different cell types: in resting cells, Akt is uniformly distributed throughout the cytoplasm and nucleus, while the membrane is only weakly stained. Stimulation of cells with growth factors such as PDGF results in rapid translocation of the protein to the membrane, followed by redistribution to the cytosol, perinuclear and nuclear regions [11, 24–26]. We examined the intracellular distribution of Akt in Saos2 cells. In resting cells Akt was mostly localized in the cytoplasm, while the membrane and the nucleus were less stained (fig. 1a). Cell treatment with PDGF resulted in a clear increase of fluorescence at the membrane, which substantially reflected the pattern described above (fig. 1b). Interestingly, staining with anti-actin (fig. 1c) suggested a colocalization of Akt protein with actin (fig. 1 d). To gain more insight into this unreported aspect of Akt, in situ cytoskeletal matrices were prepared. Cytoskeletal matrix extraction implies removal of membranes, lipids and soluble proteins by the action of detergents, digestion of DNA and high salt treatment, leaving the intact cytoskeletal filaments and the nuclear matrix [23]. During preparation, the labeling for Akt was checked after every single step (not shown), and was clearly visible after the last step (high salt extraction), as shown in figure 1 (panels e–g), clearly indicating that Akt binds strongly to the cytoskeleton, and in particular to actin (fig. 1g). Indeed, the calculated overlap coefficient between Akt and actin of 0.92 ± 0.03 strongly supported this hypothesis [21, 22].

Akt associates with actin

The above result demonstrates that Akt and actin colocalize in cytoskeletal preparations. To assess whether Akt associates to the actin skeleton, Akt was immunoprecipitated from precleared lysates of Saos2 cells. Consistent with the immunofluorescence data, actin coimmunoprecipitates with endogenous Akt (fig. 2A). Likewise, Akt was found in pellets from anti-actin immunoprecipitates, confirming that Akt and actin interact in vivo (fig. 2B). This result prompted us to investigate whether actin might be a physiological substrate of Akt. However, actin phosphorylation was not modified by Akt in an in vitro kinase assay using either recombinant or immunoprecipitated Akt and purified actin (not shown).

Previous work from different laboratories has shown that many proteins that regulate the cytoskeleton contain a PH domain and that the PH domain of signaling proteins can mediate their association to the actin fibers [27]. Moreover, recent observations have indicated that the PH domain of Akt contributes to regulate Akt activity not only by lipid binding but also by protein interaction. It was therefore

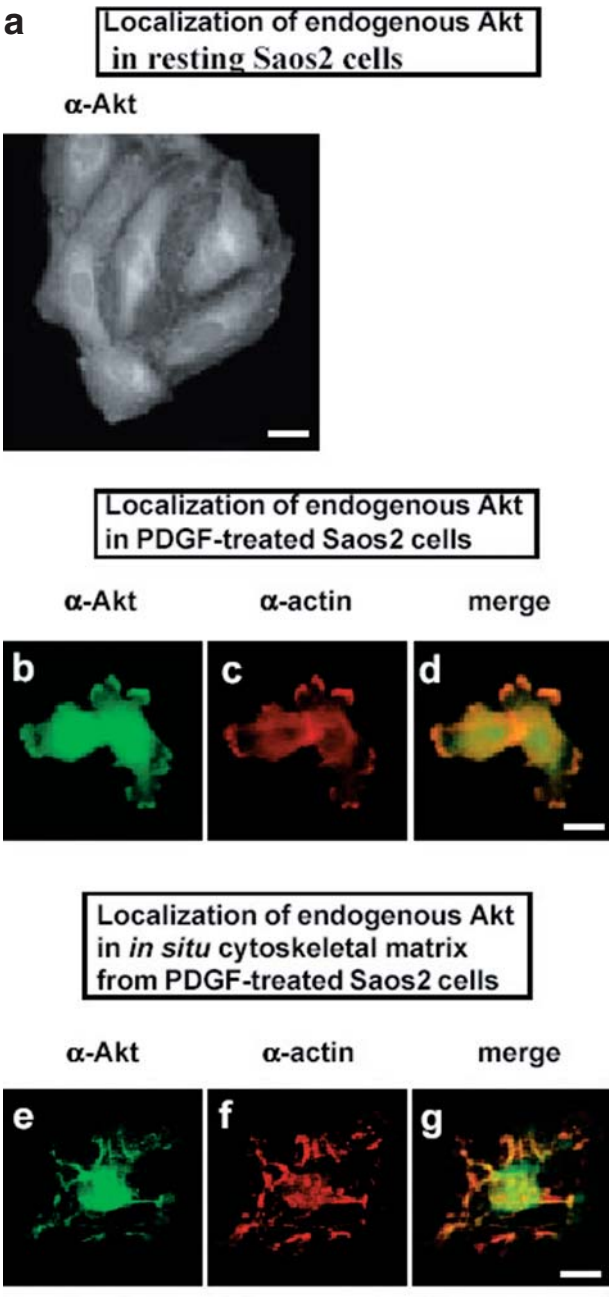

Figure 1. Colocalization of Akt with actin in in situ cytoskeletal preparation. (*a*) Saos2 cells seeded on glass coverslips were serum-starved overnight, fixed and stained with anti-Akt (New England Biolabs) to detect endogenous Akt. (*b–d*) Saos2 cells were stimulated with PDGF, fixed and double stained with anti-Akt and anti-actin (Sigma), as indicated. (*e–g*) Cells were treated in order to remove membranes, lipids, soluble proteins, DNA and other contaminants from the cytoskeleton, as described in 'Methods'. Cytoskeletal preparations were double stained as indicated, and observed by confocal microscopy. Bar, 10 µm.

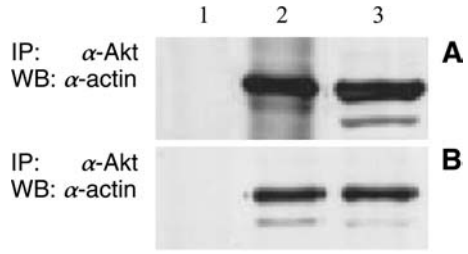

Figure 2. Association of endogenous Akt with actin in vivo and in vitro. Saos2 cells in 10% serum were lysed and immunoprecipitated (IP) with (*A*) 2 µg of anti-Akt (Santa Cruz Biotech.); coimmunoprecipitated actin was detected by immunoblot with anti-actin antibody (1:1000, Sigma); (*B*) 3 µg of anti-actin (Roche); coimmunoprecipitated Akt was detected by immunoblot with anti-Akt (1:1000, Santa Cruz). Lane 1, lysates were incubated with agarose beads only; lane 3, total lysate (60 µg). The results are representative of at least four independent experiments carried out under the same experimental conditions.

tempting to investigate whether the observed interaction between Akt and actin might be supported through the N-terminal PH domain of Akt. HEK-293 cells were transfected with Akt wild type (HA tagged) or with a plasmid encoding the amino-terminal regulatory region that spans the PH domain, called AH-Akt, HA tagged and fused to GFP [25]. Cell lysates were incubated with an anti-actin antibody and analysed by Western blotting with anti-HA, revealing the presence of Akt in the immunoprecipitates (fig. 3, lane 6). From this experiment, we conclude that the PH domain of Akt is able to associate to actin, and thus might mediate the interaction between the two proteins in vivo. Moreover, when Akt mutants carrying different degrees of truncation of the PH domain were expressed in 293 cells, Δ1-147- and Δ1-125-Akt failed to produce actin binding. Δ1-66-Akt, in which the PH domain is not completely deleted, retained actin binding ability, though to a much lesser extent than Akt wild type (fig. 3).

Since the PH domain is predominantly involved in interactions with D3-phosphoinositides, we asked whether lipid binding might affect the association of Akt to actin. However, addition of PtdIns(3,4,5)P3 to the incubation buffer did not alter Akt binding to actin, suggesting that lipid and protein binding are separate events (not shown).

Akt binds actin directly

To determine whether the interaction between the two proteins is direct, 2 µg of purified rabbit skeletal muscle actin were incubated with 8 µg of either recombinant GST-Akt, GST-AH-Akt or with glutathione-agarose beads as a control, and the blots were revealed with anti-actin antibody. Remarkably, not only full-length Akt but also the PH-containing domain AH-Akt associated in vitro with actin in the absence of other components, supporting the notion of direct binding between these proteins (fig. 4A). As a negative control, no cosedimentation of GST with actin was observed.

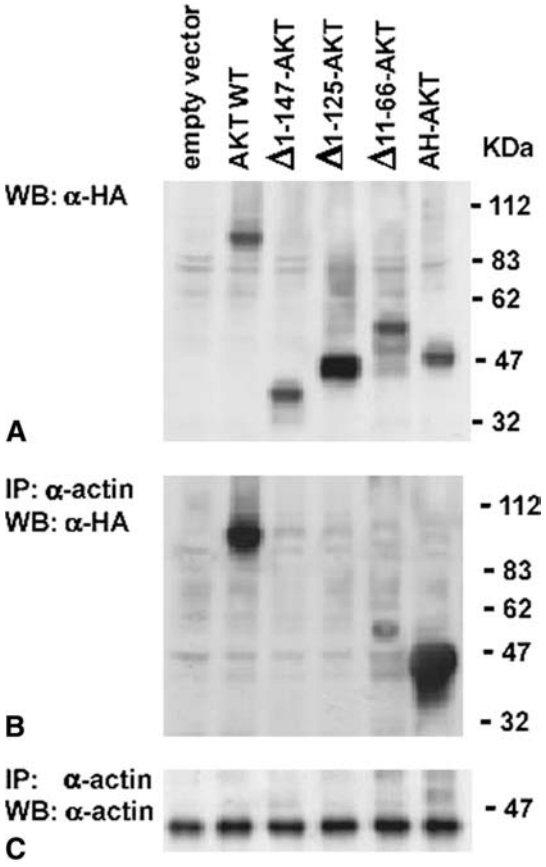

Figure 3. Akt preferentially binds actin through the PH domain. HEK 293 cells were transiently transfected with empty vector, wild-type Akt fused with EGFP (Akt wt), a plasmid encoding the PH domain of Akt fused to GFP (AH-Akt) or Akt mutants carrying three degrees of N-terminal deletion, namely Δ11-60-Akt, Δ1-125-*A*kt, Δ1-147-*A*kt. All plasmids were HA tagged. After 24 h, cells were lysed and immunoprecipitated with anti-actin. Total lysates were analysed by immunoblotting showing comparable expression of Akt constructs in all samples (*A*). Pellets from immunoprecipitation were resolved and immunoblotted with anti-HA (*B*), then reprobed with anti-actin to confirm the presence of actin in the immunoprecipitates (*C*). The result is representative of three independent experiments.

Therefore, to unequivocally settle the nature of the interaction between actin and Akt, far-Western blots for purified Akt or AH-Akt which had been separated by SDS-PAGE and transferred onto nitrocellulose were renatured and incubated with purified actin, then probed with anti-actin (fig. 4B). Different concentrations of recombinant Akt or AH-Akt were tested, ranging from 0.1 µg to 1 µg, to check for aspecific binding of the antibody. The result clearly shows that there is direct binding between actin and Akt and, more strikingly, that this binding is most probably the consequence of an interaction between actin and the PH domain of Akt (fig. 4B). The control filter, which was not incubated with purified actin, shows that the antibody does not cross-react with the recombinant proteins, even at the higher concentrations tested (fig. 4C).

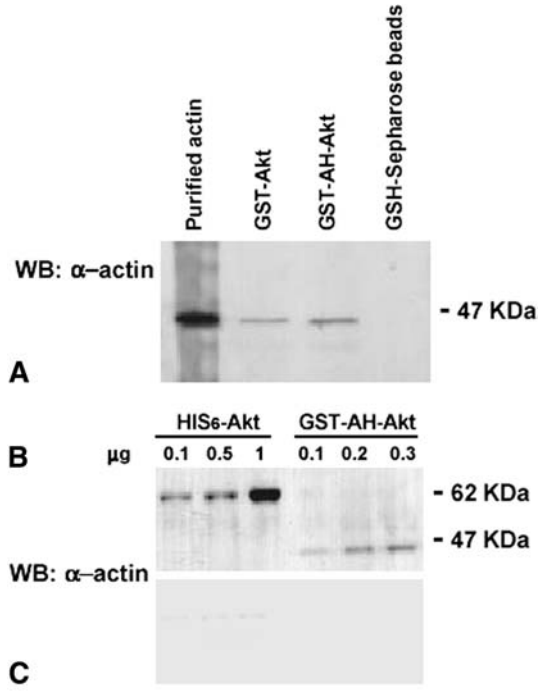

Figure 4. Akt interacts with actin directly. Bacterially expressed GST fusion proteins of Akt and AH-Akt (8 μg each) bound to GSH beads were incubated with 2 μg of purified rabbit skeletal muscle actin (Sigma) for 1 h at 4 °C. The beads were washed and analysed for actin binding by immunoblotting with anti-actin antibody. As controls, 2 μg purified actin were either loaded directly onto the gel or incubated with GST-Sepharose beads, as indicated (*A*). Increasing concentrations of human recombinant His_6-HA-Akt (0.1, 0.5, 1 μg) and GST-AH-Akt (0.1, 0.2, 0.3 μg) were loaded on a denaturing gel and blotted onto nitrocellulose filters, in double. After renaturing, filters were either overlaid with 30 μg/ml solution of purified actin in renaturing buffer (*B*), or with renaturing buffer alone (*C*), then washed and revealed with anti-actin antibody. The results are representative of two independent experiments.

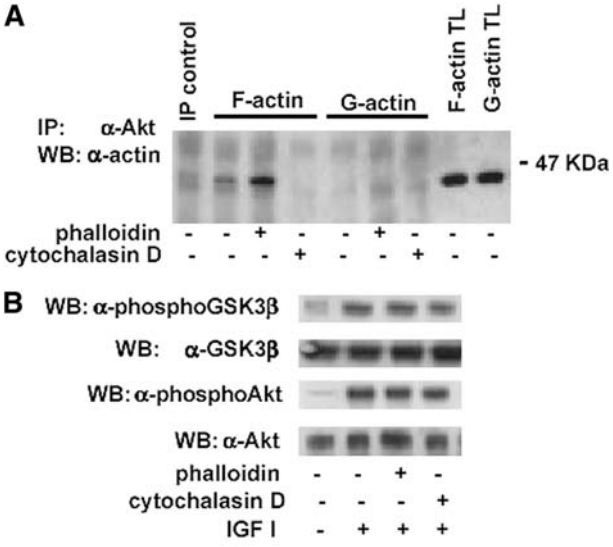

Figure 5. Akt associates with filamentous actin. (*A*) Saos2 cells were lysed in F-actin stabilization buffer, then centrifuged at 100,000 × g for 60 min. The pellet, containing F-actin, was resuspended to the same volume as the G-actin-containing supernatant; then they were immunoprecipitated with anti-Akt or with agarose beads only (IP control lane), and immunoblotted with anti-actin. Where indicated, cells were pretreated with phalloidin, to stabilize F-actin, or with cytochalasin D, to promote actin depolimerization. 50 μg of total lysates (TL) from F-actin- and G-actin-containing fractions were loaded as controls. (*B*) Saos2 cells were serum starved for 36 h, then were either left untreated or treated with IGF I (50 ng/ml) for 30 min. Where indicated, cells were pretreated with 1 μM cytochalasin D or 1 μM phalloidin. 60 μg of total lysates were resolved by SDS-PAGE and probed with anti-phosphoGSKβ or anti-phospho-Akt as noted. Then filters were reprobed with anti-GSKβ and anti-Akt to confirm equal loading.

Akt associates to filamentous actin

Having demonstrated the direct association of Akt with actin, we next asked whether Akt preferentially binds F- or G-actin. For this purpose cells were homogenized in F-actin stabilization buffer, then the two actin pools were separated by ultracentrifugation. Positive and negative controls were obtained pretreating cells with either 1 μM phalloidin, to stabilze F-actin, or 2 μM cytochalasin D, to promote actin depolimerization. The F-actin pellet was resuspended to the same volume as the G-actin-containing supernatant, and each fraction was immunoprecipitated with anti-Akt antibody and probed for the presence of actin. As shown in figure 5A, actin is clearly present only in anti-Akt immunoprecipitates from F-actin preparation. Moreover, control lanes show that pretreatment with phalloidin increases the portion of Akt bound to F-actin, while pretreatment with cytochalasin D abrogates binding.

Interaction with nonsubstrate ligands can stabilize Akt phosphorylation and activity [14]. Therefore, we examined whether actin binding can influence Akt catalytic activity. Saos2 cells were pretreated with cytochalasin D or with phalloidin, then treated with IGF I, a growth factor known to elicit phosphorylation of GSK3 by Akt. Neither cytochalasin D nor phalloidin counteracted the effect of IGF I on Akt and GSK3 phosphorylation (fig. 5B). Moreover, addition of purified actin to immunoprecipitated Akt pellets did not modify Akt activity (not shown). These data strongly suggest that binding to actin does not interfere in growth factor-induced activation of Akt, although it might be important for relaying the signal to downstream targets.

Akt phosphorylation regulates PDGF-induced targeting to actin

PDGF is known to trigger Akt phosphorylation as well as intracellular redistribution. Consequently, we asked whether PDGF might modulate the binding of Akt to actin. Indeed, though a fraction of Akt was associated to actin also in resting condition, treatment of serum-starved Saos2 cells with PDGF for 30 min led to a striking increase of this fraction, indicating that PDGF-induced Akt redistribution might be mediated through association with the actin cytoskeleton (fig. 6).

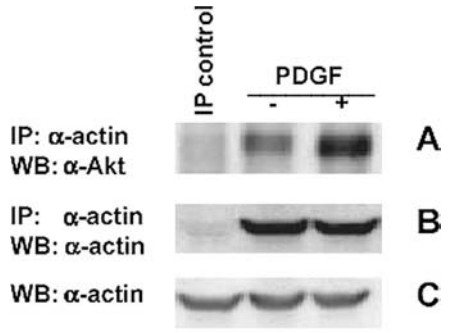

Figure 6. Effect of PDGF on Akt binding to actin. Saos2 cells were serum starved for 36 h, then were either left untreated or treated with PDGF (50 ng/ml) for 30 min. Lysates were incubated with agarose beads (IP control lane) or with anti-actin, and immunoblotted with anti-Akt (*A*). The filter was reprobed with anti-actin to confirm the presence of actin in the immunoprecipitates (*B*). Panel *C*, total lysates (30 µg) showing equal amount of protein.

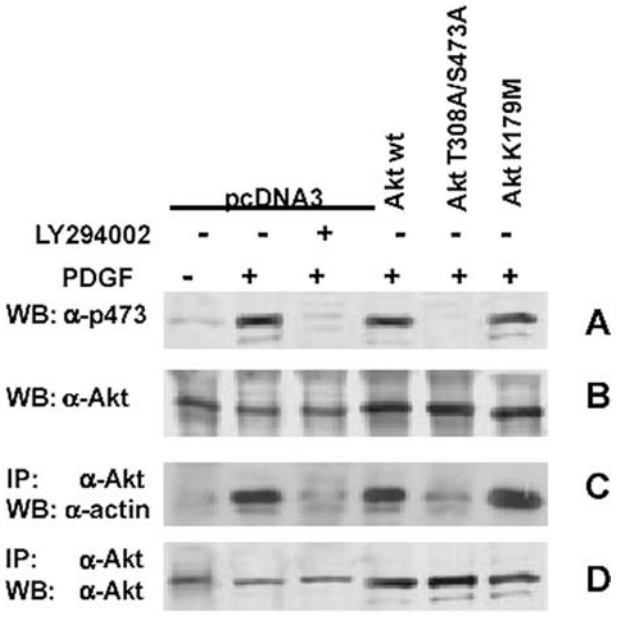

Figure 7. PDGF-enhanced actin binding requires Akt phosphorylation. HEK 293 cells were transfected with empty vector (pcDNA3), Akt wild-type (Akt wt), the mutant Akt (S473A/T308A) or the mutant Akt (K179M), as indicated. After 36 h serum starvation, cells were stimulated with PDGF for 30 min. Where indicated, cells were pretreated with LY294002 (20 µM). (*A*) Total lysates were immunoblotted with anti-phosphoSer473; (*B*) then the filter was reprobed with anti-Akt to confirm Akt expression; (*C*) lysates were immunoprecipitated with anti-Akt and revealed with anti-actin. (*D*) The membrane was reprobed with anti-Akt to confirm the presence of Akt in the immunoprecipitates.

Treatment of cells with the PI 3-kinase inhibitor LY294002 is known to strongly decrease the level of Akt phosphorylation. Therefore, cells were treated with LY294002 prior to treatment with PDGF. As expected, in cells pretreated with LY294002, PDGF failed to induce Akt phosphorylation, as detected by anti-phospho Akt (Ser473) (fig. 7A). However, a parallel decrease of the amount of actin in anti-Akt pellets was observed, suggesting that the association between Akt and actin might be regulated through Akt phosphorylation (fig. 7C). Based on the above results, we tested the ability of an Akt mutant (T308A/S473A), in which phosphorylation of the activation loop is blocked [7], to bind actin. This mutant failed to increase actin binding following PDGF stimulation (fig. 7C). It should be pointed out that the lack of reactivity of the anti-phosphoAkt-Ser473 antibody with Akt T308A/S473A is also a confirmation of the specificity of the antibody (fig. 7A, lane 5). Remarkably, Akt K179M, which has no catalytic activity but can still be phosphorylated at both S473 and T308 following PDGF stimulation, coimmunoprecipitates with actin to the same extent as wild-type Akt (fig. 7C). Taken together, the above results establish Akt phosphorylation as a pre-requisite for PDGF-dependent actin binding.

Role of Rac/Cdcd42 in Akt binding to actin

Recent work [28] demonstrated that expression of active Rac and Cdc42 induces phosphorylation of Akt both at Ser473 and Thr308, and that PDGF-induced Akt phosphorylation can be negatively modulated by dominant-negative Rac and Cdc42. Accordingly, in cells transfected with either wild-type Cdc42 or Rac, we observed an increase of endogenous Akt phosphorylation in the absence of stimuli, to the same extent observed in Saos2 cells upon PDGF treatment (fig. 8A). More important, this effect was paralleled by a concomitant increase of actin in anti-Akt pellets from wild-type Cdc42 or Rac1-overexpressing samples (fig. 8B). Conversely, expression of dominant-negative Cdc42 or Rac abrogated Akt phosphorylation even in PDGF-stimulated cells (fig. 8A). Remarkably, when Akt was immunoprecipitated from the above samples, the amount of actin associated with Akt was severely reduced compared with control cells or with wild-type Rac/Cdc42-expressing cells (fig. 8B), suggesting that Cdc42 and Rac might indeed contribute to target Akt to the actin skeleton. To further investigate the role of Cdc42 on endogenous Akt phosphorylation and actin binding, cells were transfected with GFP-actin and either wild-type Cdc42 or dominant-negative N17Cdc42. Cells were fixed and immunostained with an anti-pospho-Akt (Ser473) antibody, to detect phosphorylated Akt. When cells were transfected with GFP-actin alone, endogenous phospho-Akt was mainly compacted at the cell periphery (fig. 9B), where actin stress fibers are distributed, and essentially superimposed the actin staining, indicating that phospho-Akt colocalized with actin (fig. 9A–C). Conversely, when dominant-negative N17Cdc42 was expressed, colocalization of phospho-Akt with actin was greatly diminished, although some staining for phospho-Akt was still detectable (fig. 9D–F). Activation of Cdc42 is known to induce deep rearrangements of the actin skeleton, supporting the extension of microspikes and

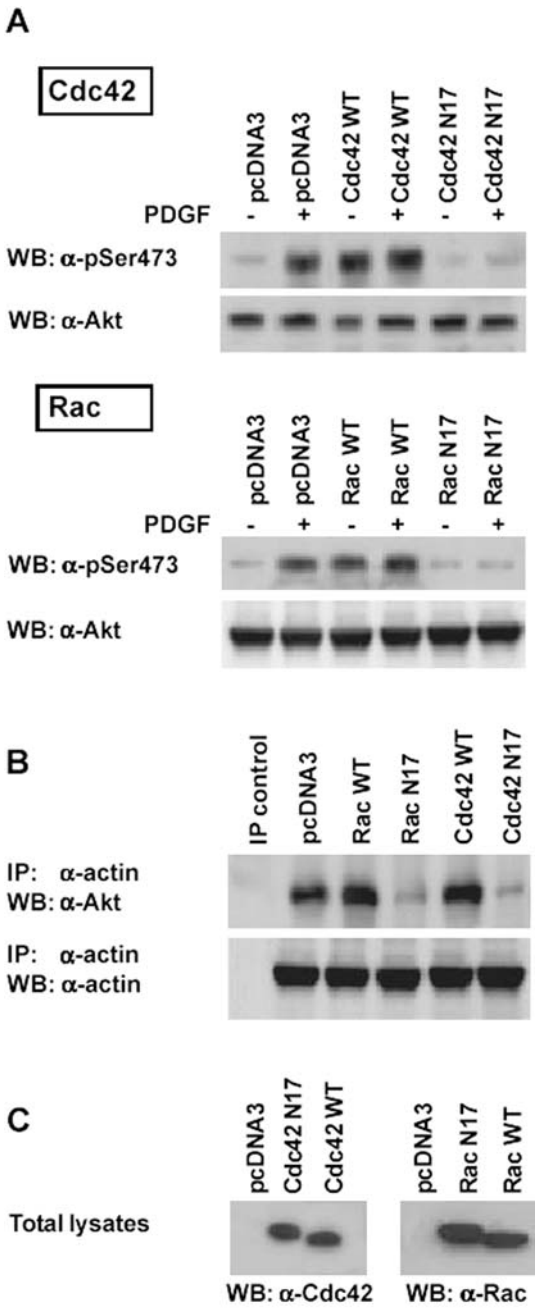

Figure 8. Rac1 and Cdc42 mediate PDGF-induced Akt recruitment to actin. 293 cells were transfected for 24 h with empty vector (pcDNA3), wild-type Cdc42 (Cdc42WT), Rac1 (RacWT) or their dominant-negative counterparts (Cdc42N17, RacN17). Serum-starved cells were either left untreated or stimulated with PDGF. (*A*) Total lysates from Cdc42 and Rac1 were immunoblotted with anti-phosphoAkt (pSer473) and reprobed with anti-Akt, as noted, to confirm equal loading. (*B*) Lysates were immunoprecipitated with agarose beads (IP control) or with anti-actin, and revealed with anti-Akt. Then the membrane was reprobed with anti-actin to confirm the presence of actin in the immunoprecipitates (lower panel). (*C*) Lysates were also checked for expression of Cdc42 by incubation with anti-Cdc42 (1:250) (lower panel, left), and Rac by incubation with anti-Rac1 (1:1000) (lower panel, right).

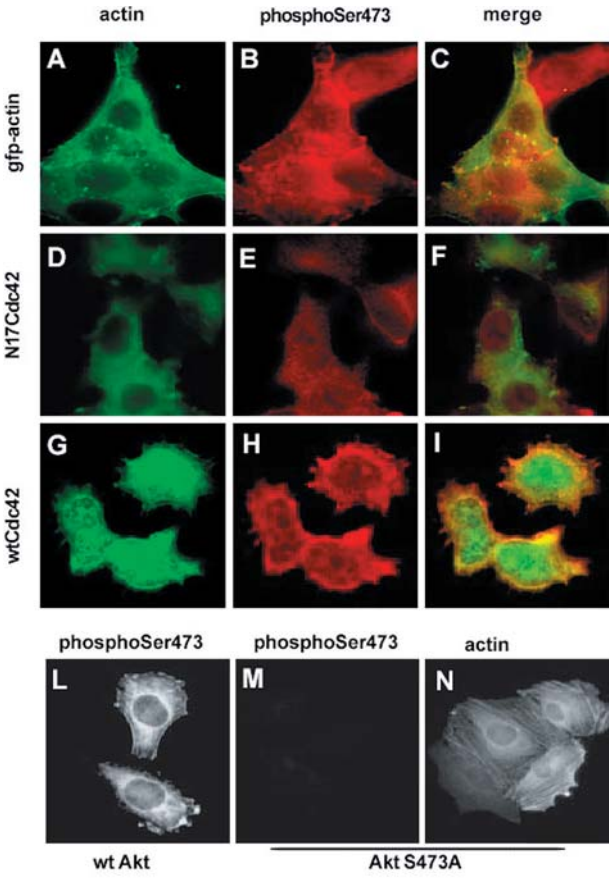

Figure 9. Active Cdc42 localizes phosphoAkt at newly formed F-actin-based microspikes and finger-like protrusions. Cycling cells were cultured onto glass coverslips and transfected with GFP-actin alone (*A–C*) or in combination with dominant-negative Cdc42 (*D–F*) or wild-type Cdc42 (*G–I*). Endogenous Akt was stained with anti-phosphoSer473 antibody. The specificity of the antibody was checked by transfecting cells with wild-type Akt (*L*), or with an unphosphorylated Akt mutant, AktS473A (*M–N*). After 24 h, the cells were fixed and stained with anti-phosphoSer473 antibody (*B, E, H, M, N*), and visualized by confocal microscopy.

filopodia and loss of stress fibers [29, 30]. The above effects were indeed evident in cells overexpressing wild-type Cdc42 (fig. 9G), indicating that wild-type Cdc42 is indeed active when expressed into cells, as confirmed by pull-down experiments (not shown). Remarkably, in this condition endogenous Akt was highly phosphorylated, as detected by staining with anti-Akt p473 (fig. 9H), and, more significantly, phospho-Akt localized predominantly to these F-actin-based membrane extensions around the perimeter of the cell (fig. 9I). The specificity of anti-phosphoAkt-Ser473 was checked transfecting cells with either wild-type Akt or an Akt mutant harbouring an alanine at position 473. It is clear from figure 9 (lower panel, L–N) that the antibody reacts with the wild-type protein but not with the unphosphorylated Akt mutant protein.

Discussion

Akt is thought to reside in the cytosol and translocate to the plasma membrane and other subcellular districts, such as the nucleus, upon cell stimulation [11, 24, 25]. One of the best-characterized stimuli that triggers both activation and intracellular redistribution of Akt is PDGF. Remarkably, it has been demonstrated that this response requires an intact cytoskeleton: disruption of the actin cytoskeleton by cytochalasin D results in a significant loss in activation of Akt [31]. Here we demonstrate that Akt binds the actin skeleton. Although we cannot rule out the possibility that a third molecule, which copurifies with Akt, mediates its interaction with actin, our data indicate that this interaction is direct, as shown by overlay as well as pull-down assays, and does not modify actin phosphorylation. Furthermore, expression of Akt mutants carrying different degrees of N-terminal truncation shows that this association is likely to be mediated by the N-terminal PH domain of Akt. More significantly, we show that this process can be modulated by PDGF. Indeed, PDGF triggers both Akt phosphorylation and a concomitant increase of the actin-bound Akt fraction. Interestingly, pretreatment of cells with LY294002, which abrogates PDGF-induced Akt phosphorylation, decreases binding to actin as well. Moreover, expression of the unphosphorylated mutant Akt T308A/S473A prevents this association, while expression of the kinase dead mutant Akt K179M, which is devoid of catalytic activity but can still be phosphorylated at S473 and T308, does not influence Akt interaction with actin. The above data strongly suggest that Akt phosphorylation facilitates the PH domain-mediated recruitment of Akt to the cytoskeleton, while the catalytic activity is dispensable. We therefore hypothesize that the PH domain might serve Akt at two separate steps: (i) it allows Akt membrane binding and phosphorylation upon cell stimulation and (ii) it mediates the interaction with actin once Akt detaches from the membrane in its phosphorylated, active conformation. Indeed, although the PH domain is known to drive the recruitment of Akt from the cytosol to the plasma membrane through its interaction with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2, it has been shown to mediate not only protein/lipid interactions but also protein/protein interactions [27, 32]. According to the available data, Akt complexes not only with substrate proteins but also with nonsubstrate ligands [14–18]. Interaction with some of these ligands, which seem to be important to regulate Akt activity as well as intracellular partitioning, are mediated through the PH domain. For instance, it has been observed that in 293 cells Akt interacts with the 14-kDa protein Tcl1, forming Tcl1-Akt high molecular weight protein complexes [17]. This interaction, which enhances Akt phosphorylation and activity, occurs in the cytoplasm through the PH domain, but leads to the translocation of Akt to the nucleus [16, 17]. In this context, Tcl1 may act as a direct transporter of Akt or may contribute to the formation of a complex that promotes the nuclear transport of Akt [17]. Very recently, the plakin family member of cytolinkers periplakin has been shown to bind the PH domain of Akt and act as a localization signal for Akt to find out its correct cellular compartment [18]. All together, these different experimental models strongly support our observation that phosphorylated Akt interacts with actin through the PH domain.

Although at first Akt and Rac/Cdc42 had been suggested to lie on parallel pathways [33], it was recently demonstrated that Rac/Cdc42 can regulate Akt activity [28, 34, 35] and that Akt participates in the regulation of cell motility downstream of Rac and Cdc42 [28, 36, 37]. We report here that in serum-starved, unstimulated Saos2 cells, expression of either wild-type Rac or Cdc42 does enhance Akt phosphorylation to the same extent as PDGF stimulation of untransfected cells, while their dominant-negative counterparts neutralize PDGF-induced phosphorylation, confirming the above-described link between these Rho GTPases and Akt. More significantly, we demonstrate that dominant-negative Rac and Cdc42 completely prevent PDGF-stimulated association of Akt to actin.

Activation of Cdc42 is known to trigger the formation of microspikes and filopodia. Remarkably, when we analysed the intracellular partitioning of phospho-Akt in cells overexpressing Cdc42, we observed that its localization was restricted to these new cytoskeletal structures, overlapping F-actin fibers. A role for Cdc42 in regulating the intracellular partitioning of signaling proteins had already been demonstrated by Coghlan et al. [38]. Indeed, investigating the translocation of PKCλ from the nucleus (where it primarily resides) to the cytoplasm in response to stimuli, these authors found that Cdc42 associates with PKCλ and confines it to the cytoplasm. Although we did not detect an interaction between Akt and Cdc42 (not shown), our data suggest that Cdc42 facilitates Akt intracellular redistribution and targeting to the actin skeleton by increasing Akt phosphorylation. But the detailed mechanism needs to be unravelled.

Filamentous actin is a continuous, dynamic structure reaching all cell sites. In our model, based on the above data, Akt docking on actin would help its intracellular redistribution to sites of interaction with other components of the signal transduction machinery, in the spatial and temporal organization which is a critical step for both efficiency and specificity of signaling [39, 40]. Ultimately, this might provide insights for the emerging role of Akt in the regulation of actin remodeling [41, 42].

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