Akt mediates Rac/Cdc42-regulated cell motility in growth factor-stimulated cells and in invasive PTEN knockout cells

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Growth factors promote cell survival and cell motility, presumably through the activation of Akt and the Rac and Cdc42 GTPases, respectively [1, 2]. Because Akt is dispensable for Rac/Cdc42 regulation of actin reorganization, it has been assumed that Rac and Cdc42 stimulate cell motility independent of Akt in mammalian cells [3–5]. However, in this study we demonstrate that Akt is essential for Rac/Cdc42-regulated cell motility in mammalian fibroblasts. A dominant-negative Akt inhibits cell motility stimulated by Rac/Cdc42 or by PDGF treatment, without affecting ruffling membrane-type actin reorganization. We have confirmed a previous report that Akt is activated by expression of Rac and Cdc42 [6] and also observed colocalization of endogenous phosphorylated Akt with Rac and Cdc42 at the leading edge of fibroblasts. Importantly, expression of active Akt but not the closely related kinase SGK is sufficient for increasing cell motility. This effect of Akt is cell autonomous and not mediated by inhibition of GSK3. Finally, we found that dominant-negative Akt but not SGK reverses the increased cell motility phenotype of fibroblasts lacking the PTEN tumor suppressor gene. Taken together, these results suggest that Akt promotes cell motility downstream of Rac/Cdc42 in growth factor-stimulated cells and in invasive PTEN-deficient cells.

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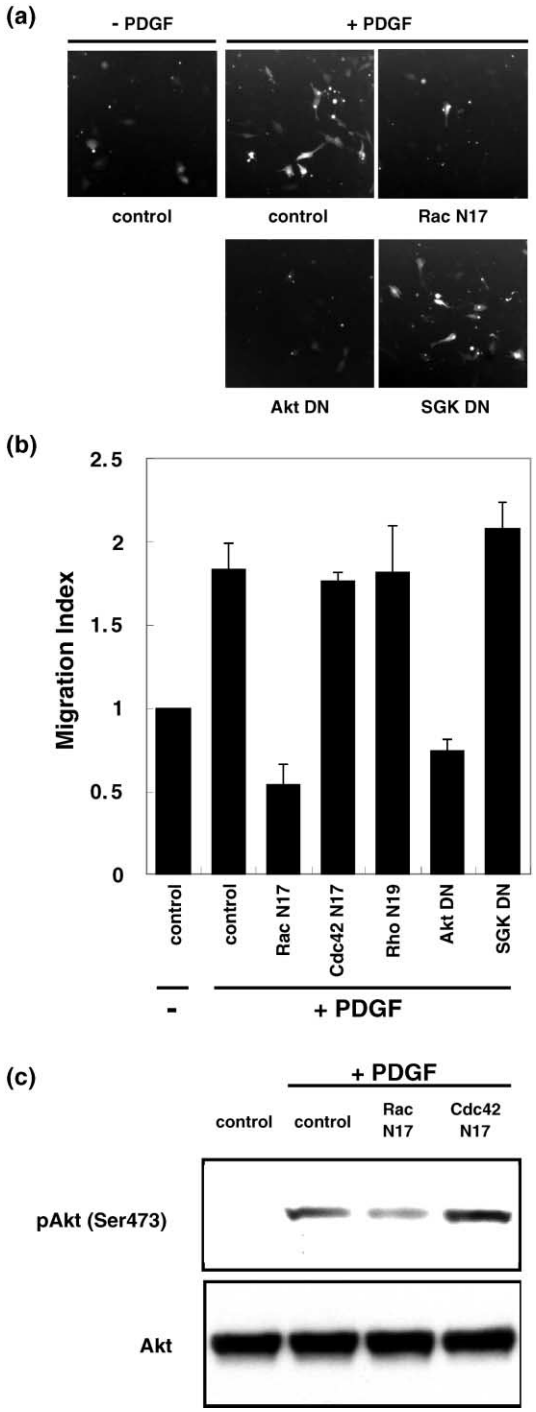
Results and discussion

To examine which molecules are involved in cell motility, we employed the "modified" Boyden chamber assay, which enables us to count GFP-labeled cells migrating across a fluorescence-blocking planar micropore membrane in real time [7, 8]. In this assay, PDGF-stimulated cell motility was suppressed by expression of a dominant-negative Rac (Rac N17) but not dominant-negative Cdc42 (Cdc42 N17) nor Rho (Rho N19) (Figure 1a,b), consistent with previous reports [9, 10]. Importantly, we found that expression of a dominant-negative mutant (K197A/T308A/S473A) of Akt resulted in marked reduction of PDGF-stimulated cell motility (Figure 1a,b). Under the same conditions, expression of the dominant-negative Akt did not affect membrane ruffling induced by PDGF (data not shown), as previously described in other systems [3, 4]. Therefore, Akt is necessary for PDGF-stimulated cell motility without affecting membrane ruffling. This is consistent with a previous report showing that Schwann cells expressing K197M-Akt were resistant to IGF-I stimulation of cell motility [11].

Because both Rac and Akt are necessary for PDGF-induced cell motility, it is possible that these molecules interact functionally. Previous reports have shown that Rac and Cdc42 can be an upstream activator of Akt in mast cells and T lymphocytes [6]. We found that expression of active Rac (Rac V12) or Cdc42 (Cdc42 V12) similarly induced phosphorylation of Akt in NIH3T3 and COS-1 cells, on both T308 and S473, the phosphorylation sites critical for activation (Figure 2a and see the Supplementary material available with this article online). In addition, expression of Rac N17 but not Cdc42 N17 partially inhibited phosphorylation of Akt in PDGF-treated fibroblasts (Figure 1c). These results taken together suggest that Rac and Cdc42 are capable of inducing phosphorylation of Akt and that PDGF induces phosphorylation of Akt at least in part through Rac in fibroblasts.

Since Rac and Cdc42 induce phosphorylation of Akt in fibroblasts, we examined whether phosphorylated Akt colocalizes with Rac and Cdc42 in these cells. A previous report has shown that phosphorylated Akt is localized at membrane ruffles [12]. We observed that phosphorylated Akt was localized predominantly at the leading edge of polarized cells, where Rac and Cdc42 were colocalized (Figure 2b). When the GFP-fused Akt PH (pleckstrin homology) domain (GFP-PHAkt) was expressed in these cells, it was also localized at the leading edge of polarized

Figure 1



Inhibition of Akt prevents PDGF-stimulated cell motility of Rat1 fibroblasts. Cells were cotransfected with a GFP-expressing vector (pEGFP) and either control vector (pcDNA3), Rac N17, Cdc42 N17, Rho N19, Akt DN (K197A, T308A, S473A), or SGK DN (T256A, S422A) for 20 hr. **(a,b)** Cells were transferred to the upper compartment of the modified Boyden chamber [7] in the presence (+) or absence (−) of 20 ng/ml PDGF. After 2 hr, the GFP-positive cells that had migrated through the membrane were counted. Typical images of migrated cells are shown in (a). Results are presented as mean ± SD of at least four independent experiments in (b). **(c)** Wild-type Akt was cotransfected with each of the indicated constructs for 18 hr. After 30 min of PDGF treatment, each set of NIH3T3 cells was harvested and subjected to immunoblotting with anti-phospho Akt(Ser473) or anti-Akt antibody.

fibroblasts (Figure 2d), consistent with the recent finding that GFP-PHAkt is localized at the lamelipodia of neutrophils [13]. Therefore, in fibroblasts, active Akt appears to localize at the leading edge with Rac and Cdc42.

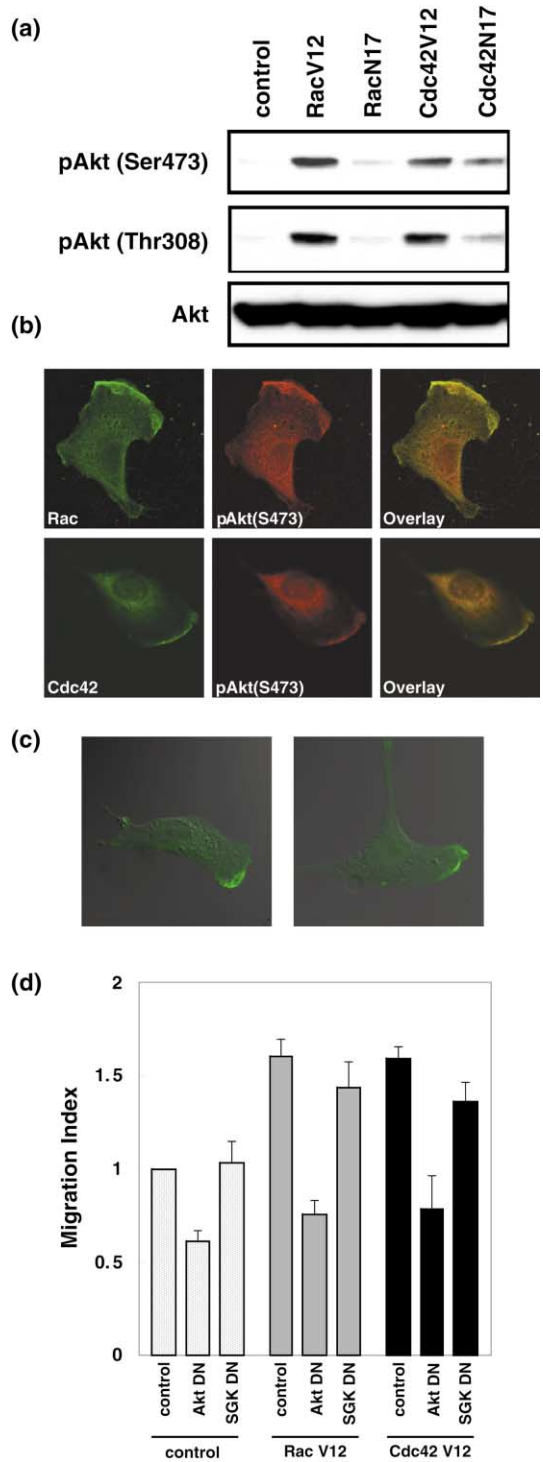
Because both Rac and Akt are necessary for PDGF-stimulated cell motility (Figure 1) and because Rac and Cdc42 induce phosphorylation of Akt (Figure 2), we next asked whether Akt is required for Rac- and Cdc42-stimulated cell motility. Expression of Rac V12 or Cdc42 V12 stimulated cell motility (Figures 2d, and 3a,b) [14]. Coexpression of dominant-negative Akt suppressed cell motility stimulated by active Rac and Cdc42 to the basal levels (Figure 2d), suggesting that Akt is an essential downstream effector of Rac and Cdc42 in stimulating cell motility.

Since Akt appears to be required for PDGF- and Rac/Cdc42-stimulated cell motility, we then asked whether Akt is sufficient for stimulation of cell motility. Expression of an active form of Akt promoted cell motility to a greater degree than expression of active Rac (Figure 3ab). This effect of Akt was cell autonomous (Figure 3c). In contrast, expression of active Akt had little effect on membrane ruffling and reorganization of the underlying actin cytoskeleton (data not shown) [3–5]. Therefore, Rac/Cdc42 may utilize two separate pathways to promote cell motility; one is an Akt-dependent pathway, and the other is a pathway leading to actin reorganization (Supplementary Figure S7). This model is further supported by the result that dominant-negative Rac inhibited Akt-stimulated cell motility only partially (see Supplementary material).

PI3-K and its downstream effector PDK1 activate a number of molecules in addition to Akt, including SGK. SGK is activated by PDK1-mediated phosphorylation and has substrate specificity very similar to Akt [15]. It is therefore possible that SGK also mediates PI3-K-stimulated cell motility. However, expression of an active SGK did not affect cell motility (Figure 3a,b). Moreover, dominant-negative SGK did not inhibit PDGF- or Rac/Cdc42-stimulated cell motility (Figures 1ab and 2d). These results suggest that Akt but not SGK promotes cell motility downstream of the PI3-K-PDK1 pathway.

Dictyostelium Akt (DdPKB) is necessary for establishing cell polarity and for chemotaxis, through the direct phosphorylation of DdPAK [16, 17]. This mechanism does not appear to be conserved between the species, because the Akt phosphorylation site on DdPAK is not conserved in

Figure 2



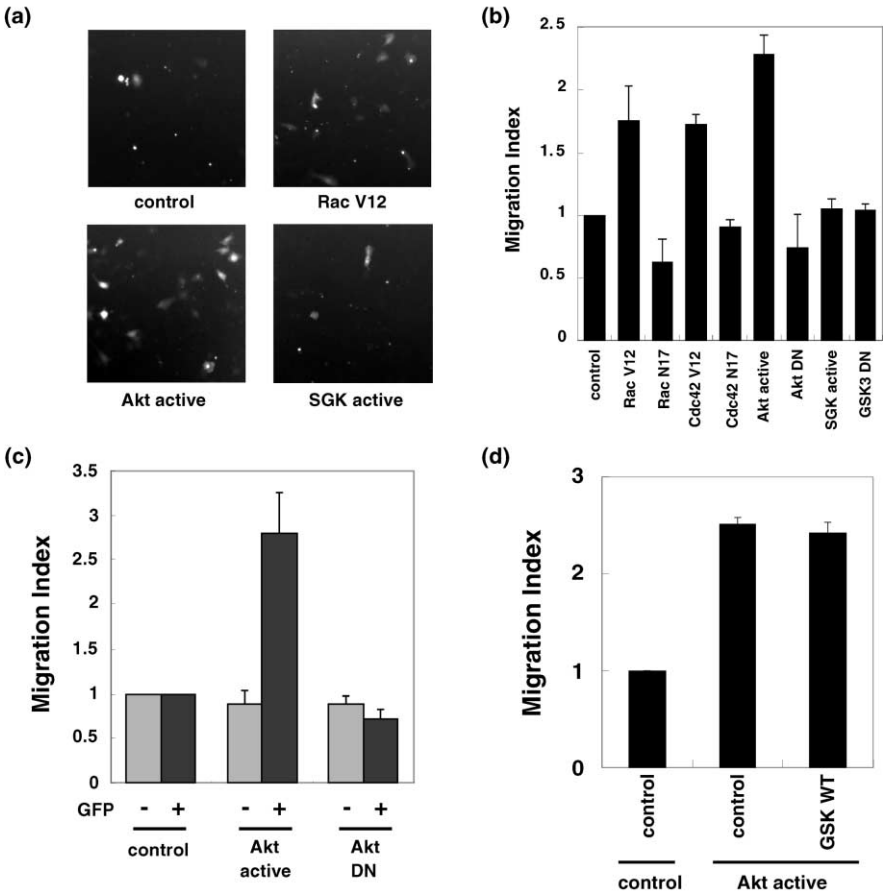
(a) Rac and Cdc42 induce phosphorylation of Akt. NIH3T3 cells were transfected with each of the indicated constructs, together with wild-type Akt (Akt WT) for 18 hr. Cell lysates were subjected to immunoblotting with anti-phospho Akt (Ser473 or Thr308) or anti-Akt antibody. **(b)** Phosphorylated Akt colocalizes with Rac/Cdc42 at the leading edge of polarized fibroblasts. NIH3T3 cells were plated on coverslips and cultured for 20 hr. After fixation in 10% trichloroacetic acid on ice, cells were stained with antibodies against phospho-Akt (Ser473) or Rac/Cdc42. **(c)** NIH3T3 cells were transfected with GFP-PHAkt for 20 hr. Cells were replated on a poly-D-lysine-coated glass-bottomed dish and cultured for 6 hr, then GFP fluorescence was detected. **(d)** Inhibition of Akt prevents Rac/Cdc42-stimulated cell motility of Rat1 fibroblasts. Cells were transfected with the indicated constructs for 20 hr. Migration was quantified 2 hr after transfer to the Boyden chamber.

mammalian PAKs and also because the localization of DdPAK at the posterior end of a moving cell is distinctly opposite of the localization of mammalian PAKs at the leading edge [16]. In addition, overexpression of DdPKB reduces cell motility [17]. In contrast, our results demonstrate that overexpression of mammalian Akt promotes cell motility. Given these results, how does Akt promote cell motility? It has recently been shown that inhibition of GSK3 downstream of PI3-K induces CLASP redistribution to the distal ends of microtubules, which stabilizes the microtubules and may contribute to the establishment of cell polarity [18]. Since Akt is known to inhibit GSK3 [19], we examined whether inhibition of GSK3 can mimic the effect of Akt. Expression of a dominant-negative GSK3 (GSK3 DN) did not promote cell motility (Figure 3b). Also, coexpression of wild-type GSK3 (GSK3 WT) did not block the stimulating effect of Akt on cell motility (Figure 3d). Therefore, it is unlikely that GSK3 serves as the primary target for Akt in regulating cell motility.

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene which is deleted or mutated in a variety of primary human cancers [20]. PTEN dephosphorylates PI(3,4,5)P_3, a product of PI3-K, and antagonizes the effects of PI3-K on apoptosis and cell migration [21]. Recently, it has been shown that PTEN negatively controls cell motility by downregulating Rac and Cdc42 [22]. Since our data suggest that Akt acts downstream of Rac and Cdc42 in the regulation of cell motility, we examined whether Akt mediates the upregulation of cell motility in the PTEN-deficient cells. We confirmed that phosphorylation of Akt is elevated in the PTEN-deficient mouse embryonic fibroblasts (*PTEN*$^{-/-}$ MEFs) compared to the level in *PTEN*$^{+/-}$ MEFs (Figure 4b) and that the cell motility of the PTEN-deficient MEFs is higher than that of *PTEN*$^{+/-}$ MEFs (Figure 4a). When a dominant-negative Akt was introduced into the PTEN-deficient MEFs, the motility was markedly reduced (Figure 4a), whereas a dominant-negative SGK had no effect (Figure 4a). Therefore, the elevated activity of Akt in the PTEN-deficient MEFs is essential for their increased cell motility.

In conclusion, we have shown that mammalian Akt plays an essential role in promoting cell motility in growth factor-stimulated cells and in PTEN-deficient cells (Supplementary Figure S7). Since Akt itself is an oncogene and may be activated in many types of cancers, it would be

Figure 3



Expression of active Akt increases motility of Rat1 fibroblasts. Cells were transfected with each of the indicated constructs, together with pEGFP, for 20 hr. The migrated cells were detected 2 hr after transfer to the Boyden chamber. **(a)** Typical images of migrated cells. **(b–d)** Results are presented as mean ± SD of at least four independent experiments. Note that active Akt (PH domain [residues 4–129]-truncated Akt with a myristoylation site) promoted cell motility. (c) Akt promotes cell motility cell autonomously. Cells were transfected with control vector, active Akt, or Akt DN, together with pEGFP, for 20 hr. All the migrated cells were labeled with DiI 2 hr after transfer to the Boyden chamber. Then, GFP-negative DiI-labeled cells (−) as well as GFP-positive DiI-labeled cells (+) were counted. Note that active Akt promoted motility of GFP-positive cells only. (d) Cells were transfected with control vector or GSK3 WT, together with active Akt, for 20 hr.

interesting to test the possibility that Akt is a common regulator of cell motility mediating increased motility and invasive characteristics of cancer cells, as well as underlying the normal enhancement of cell motility associated with wound repair, immune cell response, and various phenomena during development.

Supplementary material

Supplementary material including additional methodological detail and seven figures is available at http://images.cellpress.com/supmat/supmatin.htm.

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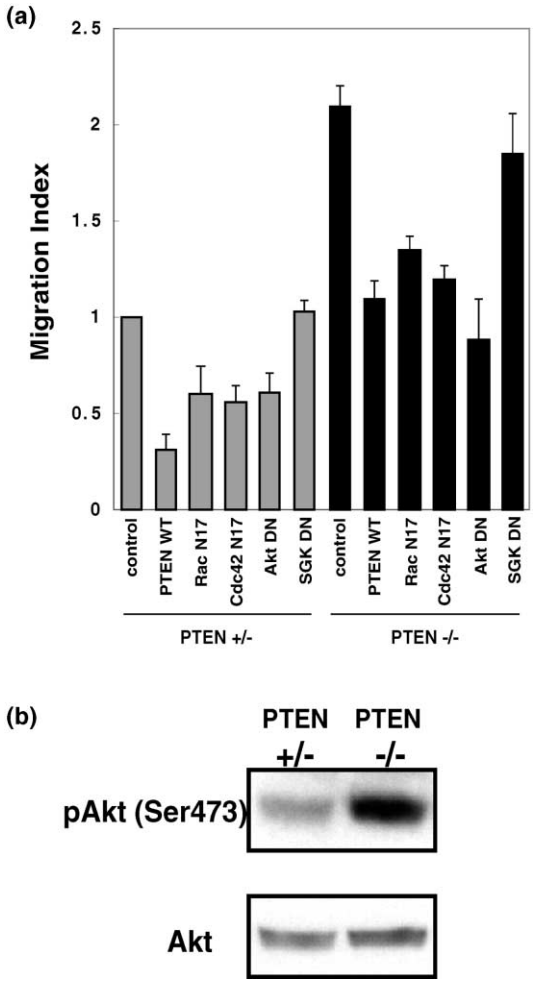
Figure 4



Increased motility of *PTEN*$^{-/-}$ cells can be reversed by expression of dominant-negative Akt. **(a)** *PTEN*$^{+/-}$ and *PTEN*$^{-/-}$ mouse embryonic fibroblasts were transfected with each of the indicated constructs, together with pEGFP, for 20 hr. Cells were transferred to the upper compartment of the modified Boyden chamber in the absence of PDGF. After 1 hr, the GFP-positive cells that had migrated through the membrane were counted. **(b)** *PTEN*$^{+/-}$ or *PTEN*$^{-/-}$ cells were cultured for 18 hr. Cell lysates were subjected to immunoblotting with anti-phospho Akt (Ser473) or anti-Akt antibody.

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