

PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration

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Qian, Yong, Linda Corum, Qiao Meng, John Blenis, Jenny Z. Zheng, Xianglin Shi, Daniel C. Flynn, and Bing-Hua Jiang. PI3K induced actin filament remodeling through Akt and p70S6K1: implication of essential role in cell migration. *Am J Physiol Cell Physiol* 286: C153–C163, 2004. First published September 10, 2003; 10.1152/ajpcell.00142.2003.—This study was designed to identify the molecular mechanisms of phosphatidylinositol 3-kinase (PI3K)-induced actin filament remodeling and cell migration. Expression of active forms of PI3K, v-P3k or Myr-P3k, was sufficient to induce actin filament remodeling to lead to an increase in cell migration, as well as the activation of Akt in chicken embryo fibroblast (CEF) cells. Either the inhibition of PI3K activity using a PI3K-specific inhibitor, LY-294002, or the disruption of Akt activity restored the integrity of actin filaments in CEF cells and inhibited PI3K-induced cell migration. We also found that expression of an activated form of Akt (Myr-Akt) was sufficient to remodel actin filaments to lead to an increase in cell migration, which was unable to be inhibited by the presence of LY-294002. Furthermore, we found that p70S6K1 kinase was a downstream molecule that can mediate the effects of both PI3K and Akt on actin filaments and cell migration. Overexpression of an active form of p70S6K1 was sufficient to induce actin filament remodeling and cell migration in CEF cells, which requires Rac activity. These results demonstrate that activation of PI3K activity alone is sufficient to remodel actin filaments to increase cell migration through the activation of Akt and p70S6K1 in CEF cells.

phosphatidylinositol 3-kinase; Rac; actin filaments

CELL MIGRATION is a key cellular feature of a wide variety of physiological and pathological processes, including embryonic development, angiogenesis, wound healing, immune response, and cell metastasis (21). It is a highly coordinated cellular event that is powered by actin filament polymerization and remodeling. The motile cells are characterized by the formation of discrete actin structures at the cell periphery for attachment to the substratum in response to extracellular signals. Those discrete actin structures are focal adhesion, stress fibers, lamellipodia, filopodia, and membrane ruffles, which are critical for adhesion, cell shape, and the generation of force (14). The processes of cell migration involve several critical steps, including the protrusion of lamellipodia and filopodia at the front of cells, the attachment of integrin to retrograde-moving actin filaments, and the contraction of actin-myosin at the rear of cells (34). However, the cellular signaling pathways that regulate these processes have not yet been fully identified.

Phosphatidylinositol 3-kinase (PI3K) is a key regulatory protein that is involved in a wide variety of cellular processes, including cell adhesion, vesicular trafficking, protein synthesis, cell growth, differentiation, cell survival, and cell transformation (24). This kinase phosphorylates the D3 position of inositol lipids and is activated by insulin, various growth factors, and cytokine. v-P3k, an active form of PI3K, was discovered as an oncogene in 1997 (8). Overexpression of v-P3k protein induces cell transformation in chicken embryo fibroblast (CEF) cells in culture, hemangiosarcomas in young chickens, and the formation of myotubes and myogenic differentiation in chicken embryo myoblast cultures (19). It has been demonstrated that PI3K is required for PDGF, TGF- β , VEGF, G protein receptors, and integrin-induced cell migration (3, 11, 18, 26, 30, 35). PI3K is required for the remodeling of actin filaments induced by growth factors (32, 38), Ras (32, 38), G protein-coupled receptor (26), and integrin (27). Stimulation of cells with insulin induces a rapid actin filament reorganization, concomitant with the recruitment of PI3K to the reorganized actin region and the activation of PI3K. Inhibition of PI3K activities abolishes insulin-induced actin filament remodeling and membrane ruffle formation (22, 23, 25, 37), suggesting that PI3K may mediate insulin-induced actin filament remodeling. Conversely, inhibition of actin filaments also affects the intracellular distribution of PI3K and the functional roles of PI3K in insulin signaling (22, 37), indicating that the integrity of actin filaments is actively involved in PI3K-mediated signal transduction. However, its role has not been fully identified in the downstream signaling of PI3K in the reorganization of actin filaments and the induction of cell motility. It has been proposed that small Rho GTPases Cdc42 and Rac might mediate the effects of PI3K on actin filaments in either insulin or integrin signaling (11, 37). In contrast, other results indicated that the activation of Cdc42 was independent of PI3K-mediated actin filament remodeling and cell migration upon stimulation of PDGF (21). Similar results were observed in opioid-induced actin filament reorganization. Stimulation of opossum kidney cells with opioid induced the activation of PI3K and the association of PI3K with PAK1 independently of the activation of Cdc42/Rac, leading to an increase in the phosphorylation and the remodeling of actin filaments (29).

In this study, we sought to dissect the downstream signaling of PI3K in remodeling actin filaments and inducing cell mi-

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gration using primary CEF cells. An active form of PI3K, v-P3k, was stably expressed in CEF cells. The experiments were designed to determine 1) whether overexpression of v-P3k was sufficient to induce actin filament remodeling and/or to increase cell migration, 2) whether Akt was required for PI3K-induced actin remodeling and cell migration, 3) whether the activation of Akt was sufficient to induce similar effects, and 4) what was the further downstream target of Akt to mediate the PI3K-induced actin remodeling and cell migration. Our study defines the roles of PI3K, Akt, and p70S6K1 signaling in the actin filament remodeling and cell migration and provides direct evidence for the interaction of these signaling molecules to mediate this process in a defined system.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), FITC-phalloidin, and tetramethylrhodamine isothiocyanate (TRITC)-anti-rabbit antibodies were purchased from Sigma (St. Louis, MO). Both phospho-Akt (Ser473) antibodies and phospho-p70S6K1 antibodies were from Cell Signaling Technology (Beverly, MA). The inhibitors, LY-294002 and rapamycin, were purchased from Calbiochem (San Diego, CA). Transwell cell migration chambers were purchased from Corning Costar (Corning, NY). Anti-Rac antibody was purchased from Upstate (Lake Placid, NY).

Plasmid constructs and cell preparation. The plasmid constructs (v-P3k, Myr-P3k, and Myr-Akt) were inserted into the avian retroviral vector, RCAS, as previously described (19). Cellular Akt with point mutations at the kinase domain (Akt-KD) and at T308A and S473A (Akt-PM) were subcloned into RCAS vector (19). A constitutively active p70S6K1 with the mutation of T389E, S411D, S418D, T421E, and S424D (CA-p70S6K1) and a dominant negative form of p70S6K1 with the inactivation of kinase (p70S6K1-KD) were subcloned into RCAS vector (9). Primary CEF cells were prepared from 10-day-old embryos obtained from SPAFAS (Preston, CT) using standard techniques (19). The RCAS constructs were transfected into CEF cells. The cells produced infectious RCAS virus that carried the insert and expressed it in cells during subsequent cycles of infection. Cells were passaged several times until all cells were infected and expressed RCAS virus.

Immunofluorescence. CEF cells were grown on cover slides. After the different treatments, cells were fixed and permeabilized as previously described (31). After being washed, cells were labeled with FITC-phalloidin for 20 min. Cells were washed again and mounted on slides with Fluoromount (Fisher, Pittsburgh, PA). A Zeiss LSM 510 microscope was used to gather images. Scales bars were generated and inserted by laser scanning microscopy software (LSM).

Western blot analysis. CEF cells were lysed in RIPA buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF] supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and 2 mM pepstatin A on ice for 30 min. After centrifugation at 14,000 rpm for 15 min, the supernatant was harvested as the total cellular protein extracts and stored at -70°C . The protein concentration was determined using Bio-Rad protein assay reagent (Richmond, VA). Aliquots of protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (vol/vol) methanol. Membranes were blocked with 5% nonfat dry milk in $1\times$ TBS containing 0.05% Tween 20 for 2 h, followed by incubation with antibodies. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (NEN, Boston, MA) and visualized with enhanced chemiluminescence reagent (NEN).

PI3K and p70S6K1 assays. CEF cells were harvested by washing with ice-cold phosphate-buffered saline (PBS) and stored at -80°C . PI3K assay was performed as previously described (20). Briefly, the cells were incubated for 15 min on ice in lysis buffer (150 mM NaCl, 100 mM Tris-HCl, pH 8.0, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 5 mM DTT, 1 mM PMSF, 1 mM sodium vanadate, 20 μM leupeptin, and 100 μM aprotinin). The lysates were cleared by centrifugation at 15,000 g for 15 min. The protein extracts in the supernatants were used for PI3K activity assay. For p70S6K1 assay, cells were lysed on ice for 40 min in 1 ml of cold immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EGTA) supplemented with 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin, 1 mM sodium orthovanadate, and 1 mM NaF. The lysates were cleared by centrifugation. The proteins (200 μg) in 300 μl of immunoprecipitation assay buffer were incubated with 1.5 μg of anti-p70S6K1 antibody (Santa Cruz Biotechnology) for 1 h at 4°C . The immunocomplex was incubated with 30 μl of protein A/G-agarose (50% slurry) (Santa Cruz Biotechnology) for 1 h at 4°C . The beads were washed two times with cold PBS, followed by one wash with assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 , and 1 mM DTT). p70S6K1 activities were measured by using a S6 assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, the beads were resuspended in 20 μl of assay dilution buffer, 10 μl of substrate, 10 μl of inhibitor mixture, and 10 μl of [γ - ^{32}P]ATP mixture (75 mM MgCl_2 , 500 μM ATP, 10 μCi of [γ - ^{32}P]ATP). The reaction mixtures were incubated for 10 min at 30°C and then centrifuged for 1 min. Aliquots (20 μl) of the supernatants were spotted onto a p81 phosphocellulose filter and washed three times for 15 min using 0.75% phosphoric acid, followed by one wash in acetone. The filters were transferred to scintillation vials and counted in a Wallac 1410 liquid scintillation counter (PerkinElmer). The data indicated means and SE values.

Cell migration assays. Cell migration assays were conducted as described (15) with a slight modification. Briefly, the cells were serum-starved overnight and the transwells were coated with enhanced chemiluminescence (ECL) cell attachment matrix (Upstate Biotechnology) at 20 $\mu\text{g/ml}$. The top chambers of transwells were loaded with 0.2 ml of cells (4×10^5 cells/ml) in serum-free media, and the bottom chambers were added with 0.6 ml of DMEM media containing 0.5% FCS. The cells were incubated in the transwells at 37°C in 5% CO_2 for 16 h. Migrating cells were fixed and stained with 0.1% crystal violet, followed by dye elution (10% acetic acid). The microplate reader was used to measure the optical density of the eluted solutions to determine the migration values. The mean values were obtained from three individual experiments and were subjected to the *t*-test.

Wound-healing assays. The wound-healing assays were performed according to the methods as described (16). Briefly, CEF cells overexpressing CA-p70S6K1 were grown on coverslips and transfected with either pGFP vector or pRK5mycRac1N17. After the cells were cultured to 100% confluent monolayer, a sterile pipette tip was used to scratch the monolayer cells to form a 100- μm "wound". The cells were then cultured in 0.5% serum media for 24 h and fixed on coverslips with formalin. The cells overexpressing GFP were stained with TRITC-phalloidin. The cells overexpressing myc-tagged Rac1N17 were incubated with an anti-c-myc antibody and then stained with both FITC-anti-mouse antibody and TRITC-phalloidin. The images were recorded using a Zeiss LSM 510 microscope. Scale bars were generated and inserted by LSM software.

Rac activation assays. Rac activation assays were performed according to the method described by Edlund (12). Briefly, the cells were washed with $1\times$ PBS supplemented with 1 mM MgCl_2 . After being washed, the cells were lysed immediately with lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl_2 , 10 $\mu\text{g/ml}$ aprotinin, and 1

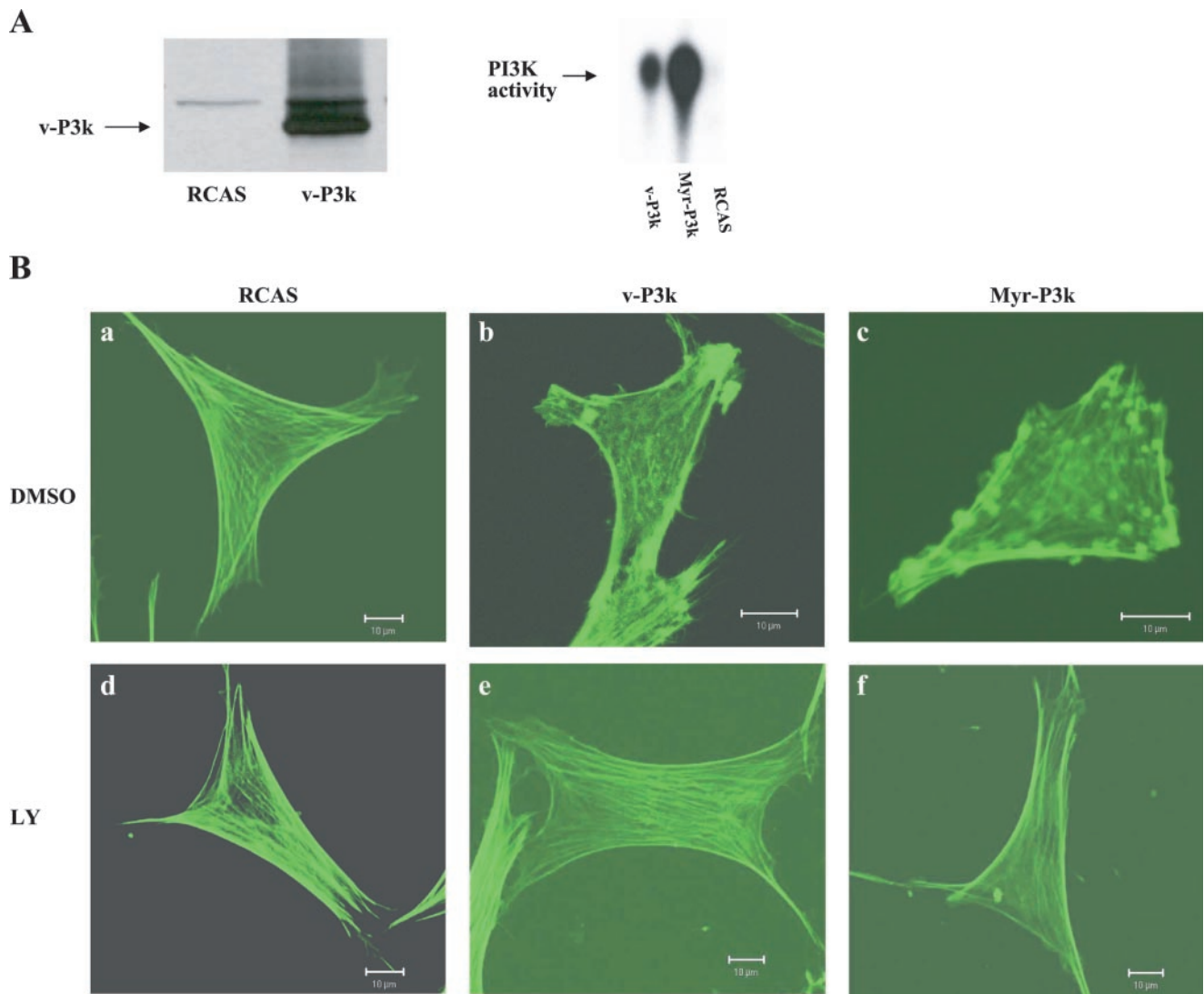


Fig. 1. Effect of constitutively active forms of phosphatidylinositol 3-kinase (PI3K) on actin filament remodeling. Chicken embryo fibroblast (CEF) cells were infected with avian retroviral vector RCAS or RCAS carrying v-P3k and Myr-P3k and cultured as previously described. *A*: protein level of v-P3k (fusion of gag and cellular PI3K) was detected by Western blotting using an anti-Gag antibody (8), and PI3K kinase activity was assayed as described (19). *B*: overexpression of constitutively active forms of PI3K is sufficient to induce actin filament remodeling. CEF cells were infected with RCAS (*a* and *d*), RCAS-v-P3k (*b* and *e*), or RCAS-Myr-P3k (*c* and *f*) and then treated with a PI3K inhibitor, LY-294002, overnight as indicated. Cells were fixed on coverslips and stained with FITC-phalloidin. Confocal microscopy was used to analyze the integrity of actin filaments and the changes in cell morphology.

mM PMSF). The lysates were centrifuged at 14,000 rpm for 15 min to obtain supernatants. The supernatants were incubated with GST-PAK-CRIB at 4°C for 20 min, and the active Rac proteins were pulled down using GST-PAK-CRIB in glutathione *S*-transferase (GST) beads. The beads were washed two times with cold wash buffer (50 mM Tris·HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 10 μg/ml aprotinin, and 0.1 mM PMSF). The Rac protein was eluted with sample buffer and subjected to 15% SDS-PAGE. The Western blot analysis was performed using an anti-Rac polyclonal antibody. Protein bands were visualized with an ECL reagent (Amersham Pharmacia).

G-actin/F-actin ratio analysis. The G-actin/F-actin ratio assay was performed according to the methods described previously (29). Briefly, the cultured CEF cells were treated with 500 μl of Triton-extraction buffer (0.3% Triton X-100, 5 mM Tris, pH 7.4, 2 mM EGTA, 300 mM sucrose, 2 μM phalloidin, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Na₃VO₄, and 50 mM NaF) for

5 min at 4°C. After the treatment, the soluble fraction was gently removed and the insoluble fraction that remained on the plates was treated with 500 μl of RIPA buffer (50 mM Tris·HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM Na₃VO₄). Both fractions were centrifuged at 12,000 rpm for 10 min at 4°C to remove the insoluble materials, followed by resolve on SDS-PAGE. The actin was detected using an anti-actin antibody (Sigma).

RESULTS

PI3K is sufficient to induce actin filament remodeling. Expression of v-P3k induces cell transformation and angiogenesis (8, 19). PI3K is required for both PDGF- and insulin-induced actin filament remodeling (22, 26). The goal of this study was to determine whether v-P3k was sufficient to induce actin filament remodeling. The CEF cells were transfected with

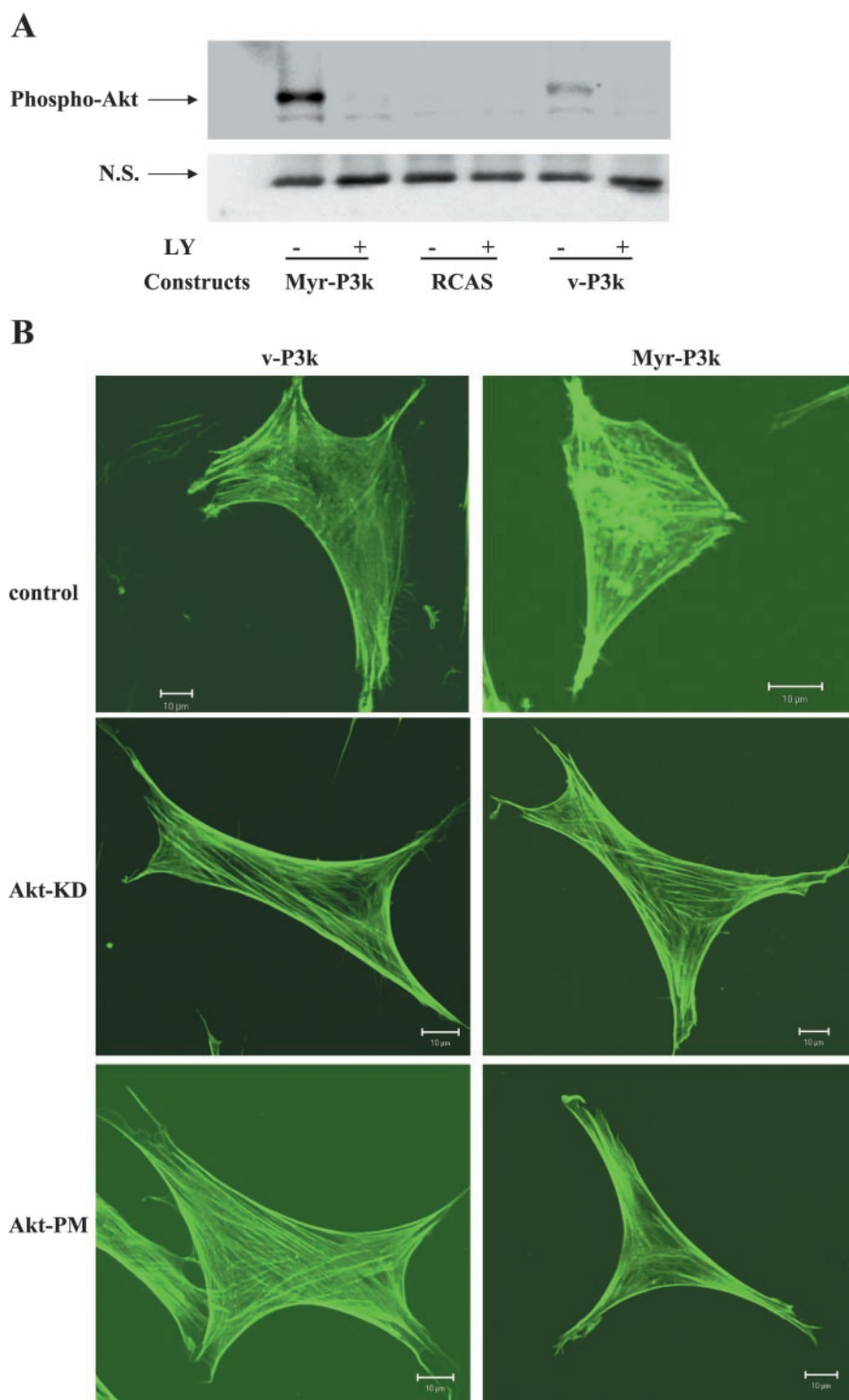


Fig. 2. PI3K remodels actin filaments through the activation of Akt. *A*: PI3K regulates the activation of Akt. CEF cells were infected with RCAS, RCAS-v-P3k, or RCAS-Myr-P3k and then incubated with LY-294002 as indicated. Cells were lysed and resolved on SDS-PAGE. The phosphorylation of Akt was detected using an anti-phospho-Akt antibody. N.S., nonspecific bands. *B*: Akt activity is required for PI3K-induced actin filament remodeling. CEF cells expressing either v-P3k or Myr-P3k were coinfecting with Akt mutants Akt-KD (Akt with the kinase death), and Akt-PM (Akt with mutations of T308A and S473A). The actin filament structure was analyzed using confocal microscopy as described above.

RCAS, RCAS-v-P3k, or RCAS-Myr-P3k, respectively. A high protein expression level of v-P3k protein was detected in the cells infected with RCAS-v-P3k (Fig. 1A), and PI3K kinase activity was greatly elevated in the CEF cells expressing RCAS-v-P3k or RCAS-Myr-P3k (Fig. 1A). To examine the morphological changes, the CEF cells were stained with FITC-phalloidin for actin filaments and analyzed by confocal microscopy. In the CEF cells infected with RCAS, actin filaments were evenly distributed and bundled actin stress fibers tra-

versed throughout the cells without significant appearance of motility structures (Fig. 1B). In the cells infected by RCAS-v-P3k, the actin filaments were redistributed into rosette-like structures, the actin stress fibers were decreased, and both lamellipodia and filopodia were increased (Fig. 1B). To test whether another active form of PI3K affects actin filaments, the CEF cells were also infected with RCAS-Myr-P3k. Similarly, expression of Myr-P3k in the cells substantially induced actin filament remodeling (Fig. 1B). Preincubation of the cells

with a PI3K inhibitor, LY-294002, blocked the effects of both v-P3k and Myr-P3k on actin filament remodeling in CEF cells (Fig. 1B). These results indicate that expression of PI3K alone is substantial enough to induce the remodeling of actin filaments to form the cell motility structures in CEF cells and that PI3K activity is required for actin filament remodeling in the cells.

PI3K remodels actin filament through the activation of Akt. Akt is one of the best-known targets of PI3K. Akt plays an important role in cell transformation and angiogenesis (1, 19). The next objective was to study whether PI3K regulated the remodeling of actin filaments through the activation of Akt. The experiments were designed to examine whether overexpression of v-P3k was sufficient to induce the activation of Akt. The cellular protein extracts from the CEF cells expressing RCAS, RCAS-v-P3k, or RCAS-Myr-P3k were analyzed by Western blot using a specific anti-phosphorylated Akt antibody. Compared with RCAS controls, both v-P3k and Myr-P3k substantially increased Akt phosphorylation (Fig. 2A). Incubation of the cells with a PI3K inhibitor, LY294002, abrogated the Akt phosphorylation induced by both v-P3k and Myr-P3k (Fig. 2A). Similarly, the kinase assays demonstrated that Akt kinase activity was induced by v-P3k and Myr-P3k and inhibited by LY-294002 in the same experiments (data not shown). These results indicate that overexpression of v-P3k is sufficient to induce the activation of Akt in CEF cells. To determine whether PI3K may regulate the remodeling of actin filaments through the activation of Akt, the dominant negative forms of Akt, Akt-KD and Akt-PM, were coexpressed with v-P3k and Myr-P3k in CEF cells. The forced expression of dominant negative forms of Akt inhibited PI3K-induced remodeling of actin filaments in CEF cells (Fig. 2B), indicating that Akt is required for PI3K-induced remodeling of actin filaments. Taken together, these results indicate that PI3K remodels actin filaments through the activation of Akt in CEF cells.

PI3K is sufficient to increase cell migration through the activation of Akt. Both lamellipodia and filopodia are cell motile structures. The increase of these structures upon overexpression of active forms of PI3K indicates that PI3K may have an ability to enhance cell migration. To test this possibility, the cell migration assays were performed. As shown in Fig. 3, the overexpression of either v-P3k or Myr-P3k was sufficient to induce an increase in cell migration (Fig. 3, bars 2 and 5). We then added an actin filament inhibitor, cytochalasin D (CD). The results showed that the inhibition of actin filaments disrupted PI3K-induced cell migration (Fig. 3, bars 4 and 7). Next, we asked whether PI3K-induced cell migration was mediated by the activation of Akt. Coexpression of a kinase-dead Akt (Akt-KD) with v-P3k substantially inhibited PI3K-induced cell migration (Fig. 3, bars 3 and 6), indicating that the activity of Akt is required for PI3K-induced cell migration. Taken together, these results indicate that PI3K remodels actin filaments, leading to an increase in cell migration through the activation of Akt in CEF cells.

Akt is sufficient to induce actin filament remodeling and cell migration. To determine whether overexpression of a constitutively active form of Akt was sufficient to induce the remodeling of actin filaments, leading to an increase in cell migration in CEF cells, a constitutively active form of Akt, Myr-Akt, was expressed in CEF cells using a RCAS vector. Expression of

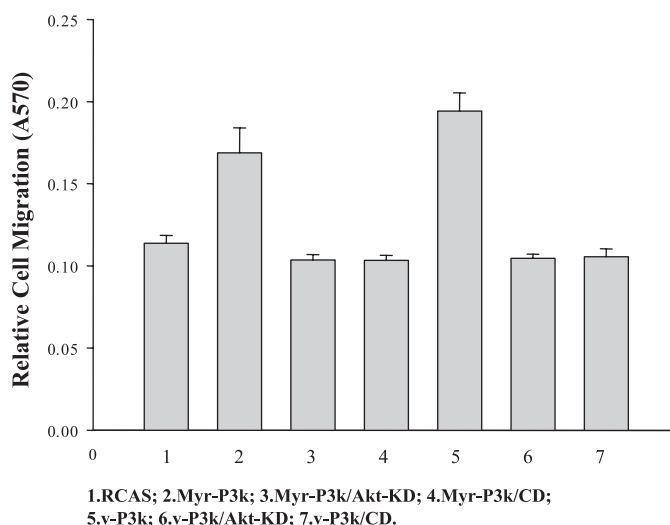


Fig. 3. PI3K induces cell migration through the activation of Akt. CEF cells expressing either v-P3k or Myr-P3k were coinfecting with Akt-KD or treated with 1 μ M cytochalasin D (CD) and subjected to the cell migration assays as described in MATERIALS AND METHODS. The results indicate means \pm SE of 3 separate assays ($P < 0.05$, $n = 3$).

Myr-Akt alone induced a profound reorganization of actin filaments, dramatically increased the formation of cell motile structures, and changed the cell morphology (Fig. 4A). These changes were not blocked by preincubation of the cells with a PI3K inhibitor, LY-294002. Protein extracts from CEF cells expressing RCAS and RCAS-Myr-Akt were analyzed by Western blot using a specific anti-phosphorylated Akt antibody. Expression of Myr-Akt significantly increased Akt phosphorylation (Fig. 4B). Although Akt phosphorylation was partially reduced in the cell expressing Myr-Akt upon the addition of LY-294002, it was still much higher compared with that of vector control (Fig. 4B). The cell migration assays demonstrated that overexpression of Myr-Akt alone was sufficient to increase cell migration, which was only partially attenuated upon the addition of LY-294002 (Fig. 4C). The inhibition of actin filaments with CD abrogated Akt-induced cell migration (Fig. 4C). Taken together, these results indicate that Akt is sufficient to induce actin filament remodeling and increase cell migration in CEF cells.

p70S6K1 is a downstream target of Akt in actin filament remodeling and cell migration. p70S6K1 has been implicated as an Akt downstream target. The next study was to test whether p70S6K1 was involved in PI3K- and Akt-mediated actin filament remodeling and cell migration. CEF cells expressing v-P3k, Myr-P3k, and Myr-Akt were used to test the phosphorylation of p70S6K1, which correlated to the activation of p70S6K1. The phosphorylation of p70S6K1 was analyzed in the absence or the presence of rapamycin, an mTOR/p70S6K1 inhibitor. It was found that p70S6K1 phosphorylation was induced by active forms of PI3K and Akt and inhibited by rapamycin (Fig. 5A). In CEF cells expressing v-P3k, Myr-P3k, and Myr-Akt, rapamycin completely restored the integrity of actin filaments as observed in RCAS-infected cells (Fig. 5B), indicating the requirement of mTOR/p70S6K1 in PI3K/Akt-induced actin filament remodeling. To rule out the possibility of inhibitory effects of rapamycin on Akt activities, v-P3k-, Myr-P3k-, and Myr-Akt-infected cells were treated

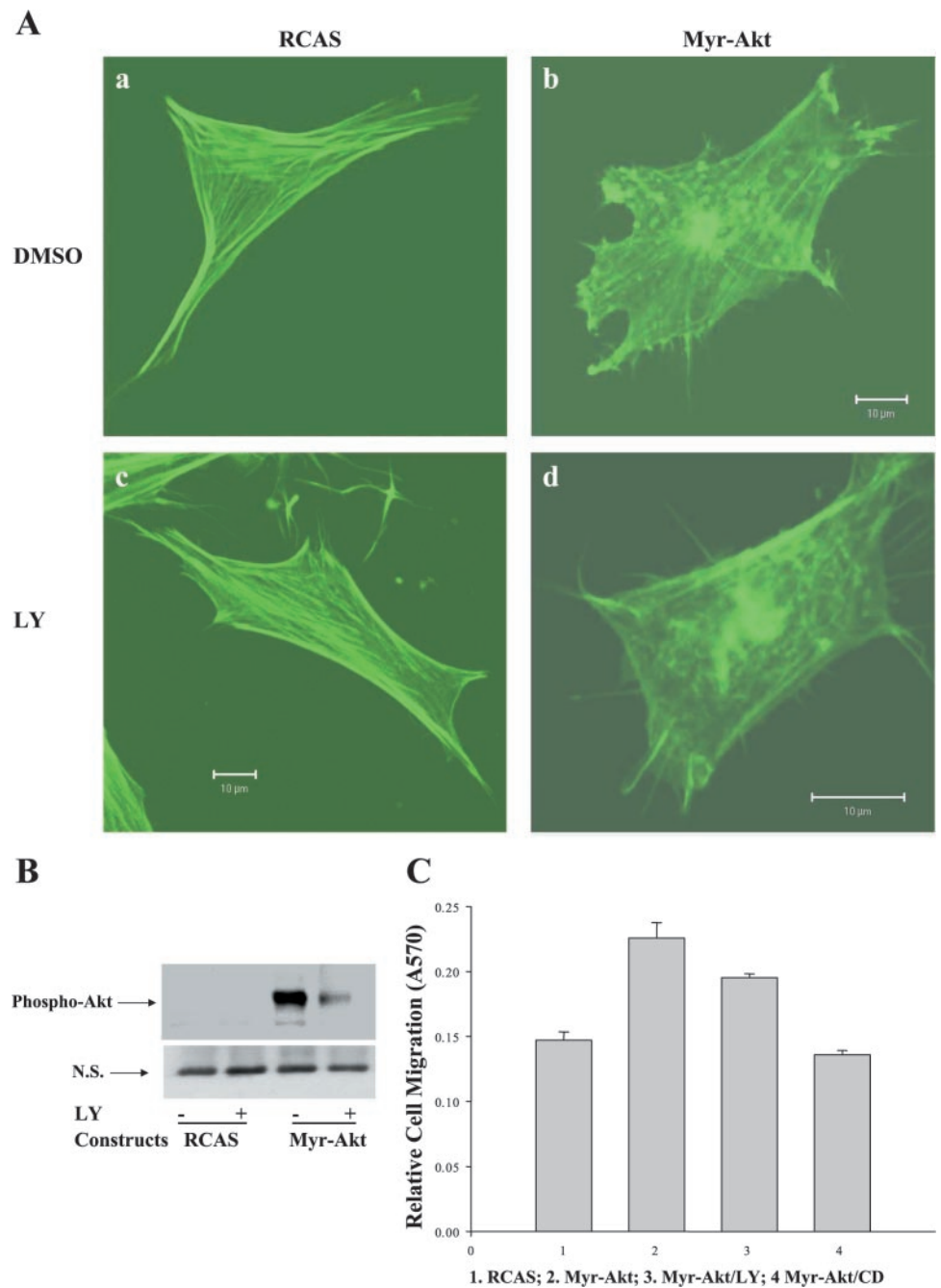


Fig. 4. Effect of a constitutively active form of Akt on actin filament remodeling and cell migration. **A:** overexpression of a constitutively active form of Akt is sufficient to induce actin filament remodeling. CEF cells were infected with either RCAS (*a* and *c*) or RCAS-Myr-Akt (*b* and *d*) and treated with a PI3K inhibitor, LY-294002 (*c* and *d*). The actin filaments were analyzed by confocal microscopy. **B:** LY-294002 partially blocked Myr-Akt-induced Akt activity. CEF cells were infected with either RCAS or RCAS-Myr-Akt and then incubated with LY-294002 as indicated. The cellular protein extracts were prepared from the cells, and the Akt phosphorylation was detected by immunoblotting using an anti-phospho-Akt antibody. N.S., nonspecific bands. **C:** overexpression of a constitutively active form of Akt is sufficient to induce cell migration. CEF cells expressing Myr-Akt were treated with either LY-294002 or CD as indicated and subjected to the cell migration assays as described in MATERIALS AND METHODS. The results indicate means \pm SE of 3 separate assays ($P < 0.05$, $n = 3$).

with rapamycin and the cell lysates were subjected to Western blot analysis using a specific anti-phospho-Akt antibody. The results showed that the incubation of the cells with rapamycin had no effects on Akt activity (Fig. 5C). To specifically inhibit p70S6K1 kinase activity, we constructed a mutant of p70S6K1 with the kinase death (p70S6K1-KD) and found that overexpression of p70S6K1-KD greatly inhibited the endogenous p70S6K1 activity (Fig. 5E), indicating that p70S6K1-KD can serve as a dominant negative protein. p70S6K1-KD was then coexpressed with v-P3k, Myr-P3k, or Myr-Akt in CEF cells. The results showed that the overexpression of p70S6K1-KD completely blocked v-P3k-, Myr-P3k-, and Myr-Akt-induced actin filament remodeling (Fig. 5B). Similar results were obtained using endothelial cells (data not shown). Inhibition of

p70S6K1 with rapamycin decreased both v-P3k- and Myr-Akt-induced cell migration (Fig. 5, F and G). Taken together, these studies indicate that p70S6K1 is an essential target of PI3K and Akt in inducing actin filaments remodeling and cell migration in CEF cells.

p70S6K1 is sufficient to induce actin filament remodeling and cell migration. To examine whether overexpression of a dominant positive p70S6K1 is sufficient to induce the changes in cell morphology and motility in CEF cells, a constitutively active form of p70S6K1, CA-p70S6K1, was expressed in CEF cells using RCAS vector. Overexpression of CA-p70S6K1 induced a significant increase in p70S6K1 kinase activity (Fig. 6A), indicating that CA-p70S6K1 is functional. The immunofluorescence analysis showed that the overexpression of CA-

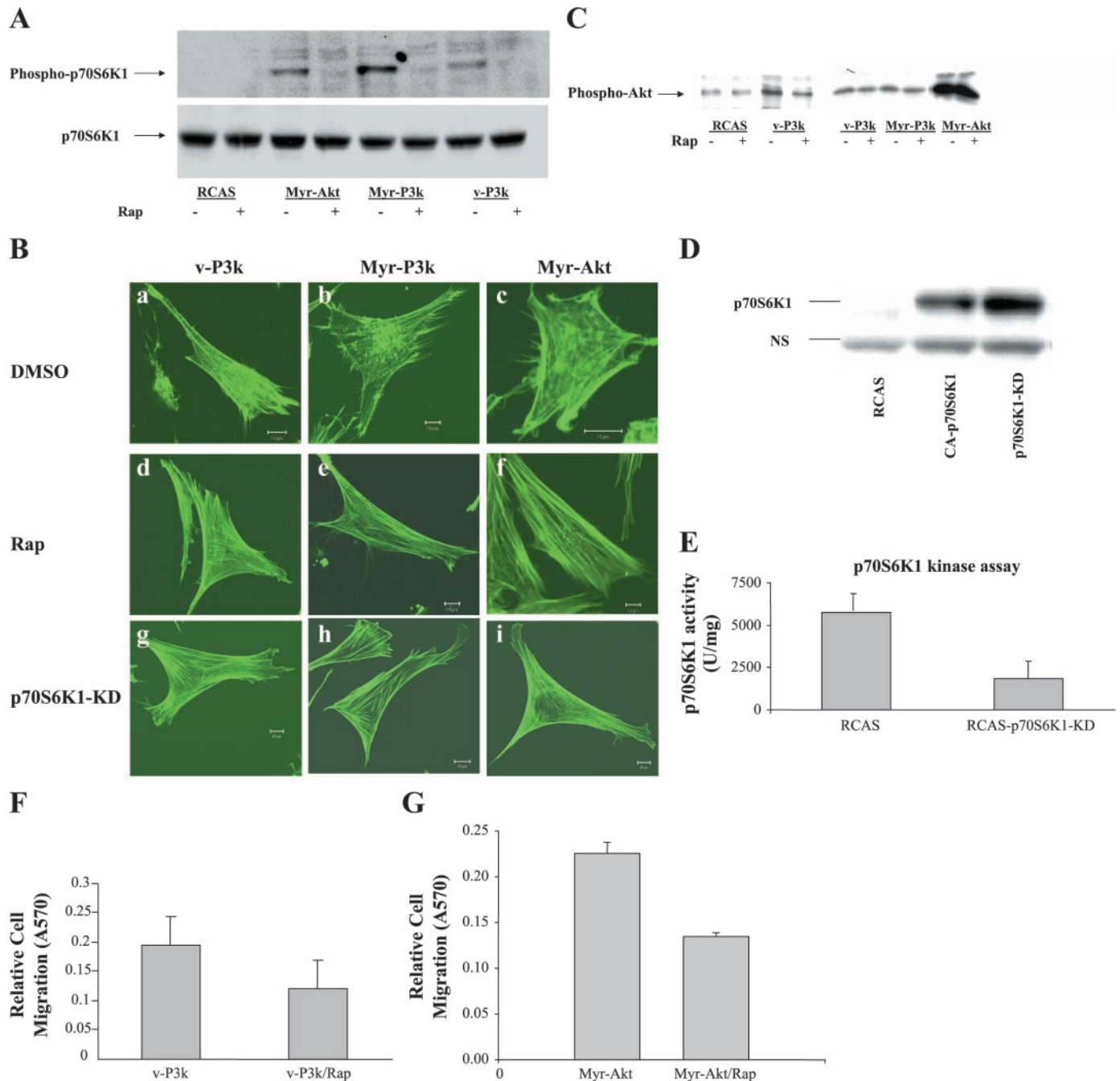


Fig. 5. p70S6K1 mediates the effects of PI3K and Akt on actin filament remodeling and cell migration. *A*: overexpression of either active form of PI3K or active form of Akt activates p70S6K1. CEF cells were infected with RCAS or RCAS carrying Myr-P3k, v-P3k, and Myr-Akt as indicated. The levels of p70S6K1 protein phosphorylation were analyzed by immunoblotting using an anti-phospho-p70S6K1 antibody. *B*: p70S6K1 activity is required for PI3K- and Akt-induced actin filament remodeling. CEF cells were infected by RCAS-v-P3k (*a, d, and g*), RCAS-Myr-P3k (*b, e, and h*), or RCAS-Myr-Akt (*c, f, and i*) and then treated with 15 ng/ml rapamycin (*d-f*) or coinfecting with p70S6K1-KD (*g-i*) as indicated. The cells treated with DMSO were used as the control. The structure of actin filaments was analyzed as described above. *C*: prolonged treatment of rapamycin had no effect on Akt activity. CEF cells infecting with either RCAS-Akt, RCAS-Myr-P3k, or RCAS-v-P3k were treated with 15 ng/ml rapamycin overnight as indicated. After treatment, cells were lysed and Akt kinase assays were performed according to the methods previously described. *D*: expression of p70S6K1 protein. The cells expressing RCAS or RCAS carrying p70S6K1 mutants were lysed and used to analyze the levels of p70S6K1 levels by immunoblotting. *E*: overexpression of a dominant negative form of p70S6K1 (p70S6K1-KD) suppresses the endogenous p70S6K1 activity. CEF cells were either infected with RCAS alone or RCAS-p70S6K1-KD as indicated. Because p70S6K1-KD was tagged at an NH₂ terminus with HA, the cellular protein extracts from the cells infected by RCAS alone or RCAS-p70S6K1-KD were incubated with anti-HA antibody, followed by the addition of protein A/G agarose to remove the forced expression of p70S6K1-KD. The endogenous p70S6K1 kinase assays were performed, and the results were plotted with Microsoft Excel ($P < 0.01$, $n = 3$). *F* and *G*: rapamycin inhibits PI3K- and Akt-induced cell migration. CEF cells expressing either v-P3k (*F*) or Myr-Akt (*G*) were pretreated with 15 ng/ml of rapamycin for 30 min and then subjected to the cell migration assays as described in MATERIALS AND METHODS. The results are means \pm SE of 3 separate assays ($P < 0.05$, $n = 3$).

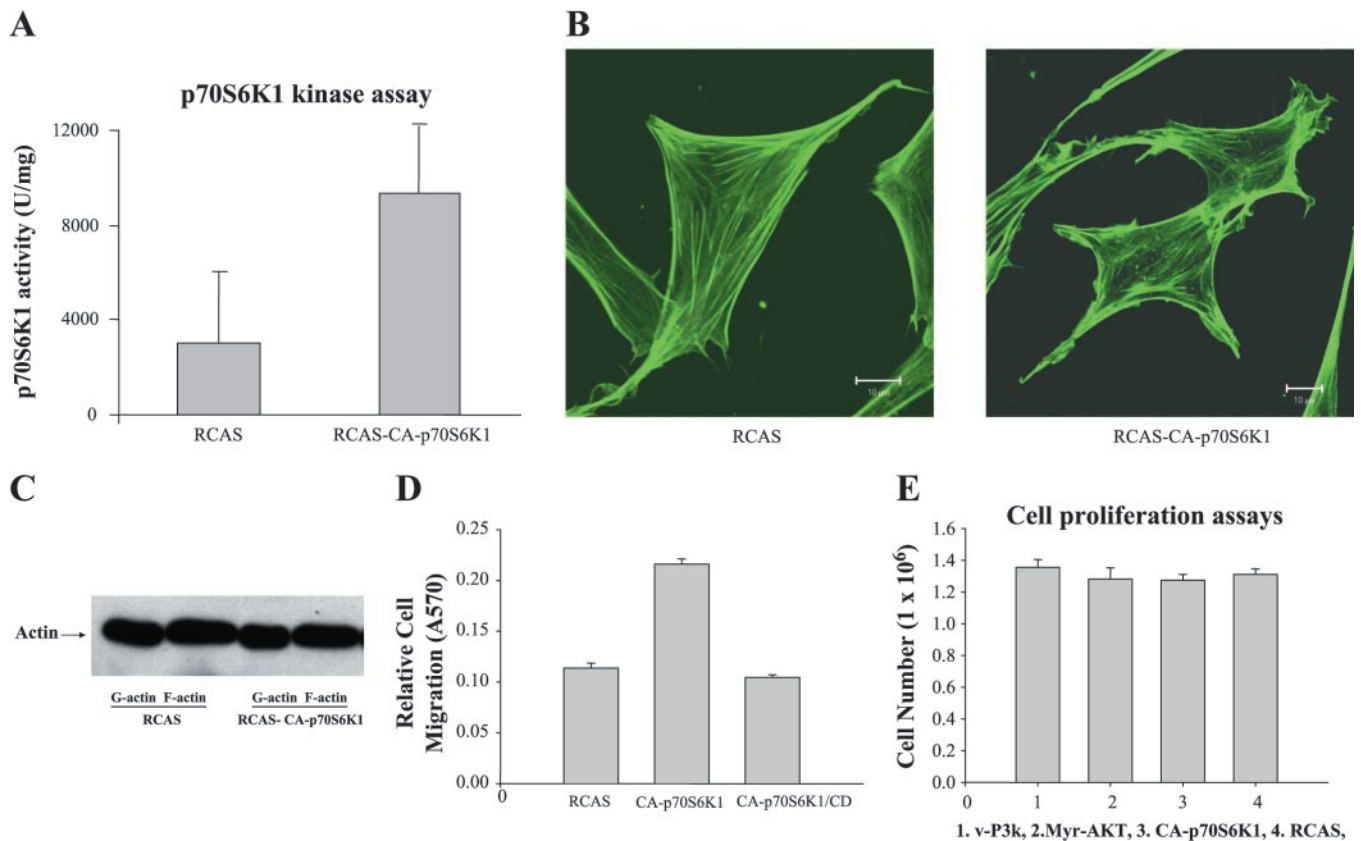


Fig. 6. Effect of a constitutively active form of p70S6K1 on actin filament remodeling and cell migration. *A*: expression of CA-p70S6K1 increased p70S6k activity. CEF cells were either infected with RCAS alone or RCAS-CA-p70S6K1 as indicated. The p70S6K1 kinase assays were performed as described in MATERIALS AND METHODS ($P < 0.01$, $n = 3$). *B*: overexpression of a constitutively active form of p70S6K1 is sufficient to induce actin filament remodeling. CEF cells were infected with RCAS (*left*) or RCAS-CA-p70S6K1 (*right*). The structure of actin filaments was analyzed by confocal microscopy. *C*: G-actin/F-actin ratio assays. CEF cells expressing RCAS vector or RCAS-CA-p70S6K1 were subjected to Triton X-100 fractionation analysis. Both G-actin (Triton-soluble fraction) and F-actin (Triton-insoluble fraction) were resolved on SDS-PAGE, followed by the immunoblotting analysis with an anti-actin antibody. *D*: overexpression of a constitutively active form of p70S6K1 is sufficient to induce cell migration. CEF cells expressing CA-p70S6K1 were subjected to the cell migration assays with or without CD treatment. The results are means \pm SE of 3 separate assays ($P < 0.01$, $n = 3$). *E*: aliquots of cells treated as above were plated and cultured for 16 h. The cell proliferation was assayed using the cells expressing RCAS vector alone or RCAS carrying various constructs as indicated. The results are means \pm SE of 3 separate assays ($P > 0.05$, $n = 3$).

p70S6K1 induced a profound actin filament remodeling in the cells. The number of both lamellipodia and filopodia was increased in the cell edge, whereas the number of actin stress fiber structures was decreased in the cell body (Fig. 6B). The increase in the structures of lamellipodia and filopodia indicates that the cross-linking of actin filaments may be enhanced to form actin bundling structures and/or the polymerization of actin is upregulated. To examine whether the overexpression of an active form of p70S6K1 would increase actin polymerization, both CEF cells expressing RCAS vector or RCAS-CA-p70S6K1 were subjected to Triton 100 fractionation. It has been well proven that G-actin exists in the Triton 100-soluble fraction and that F-actin exists in the Triton 100-insoluble fraction (13). The results of Triton 100 fractionation assays demonstrated that there was no significant difference in the G-actin/F-actin ratio between CEF cells expressing RCAS and RCAS-CA-p70S6K1, indicating that overexpression of the active form of p70S6K1 does not affect actin polymerization (Fig. 6C). Overexpression of CA-p70S6K1 significantly increased cell migration, which can be inhibited by the treatment with an

actin filament inhibitor, CD (Fig. 6D). These results indicate that the expression of CA-p70S6K1 is sufficient to induce actin filament remodeling and cell migration in CEF cells.

Rac activity is required for p70S6K1-induced actin filament remodeling and cell migration. The morphological changes in cells overexpressing CA-p70S6K1 indicate that small Rho GTPases Cdc42/Rac might be involved in p70S6K-induced cell signaling. Because Cdc42 was proven to be independent of PI3K-mediated actin filament remodeling and cell migration (21, 29), we examined whether the activation of Rac was required for CA-p70S6K-induced actin filament remodeling and cell migration in this study. The CEF cells overexpressing CA-p70S6K1 were transiently transfected with a dominant negative Rac1 or vector alone. The results showed that the dominant negative Rac1 inhibited CA-p70S6K1-induced actin filament remodeling (Fig. 7A), suggesting that Rac activity is required for the actin filament remodeling. However, the GST-PAK-CRIB pulldown assays showed that expression of active form of p70S6K1 was unable to activate Rac activities in CEF cells (Fig. 7B). To examine whether the disruption of Rac

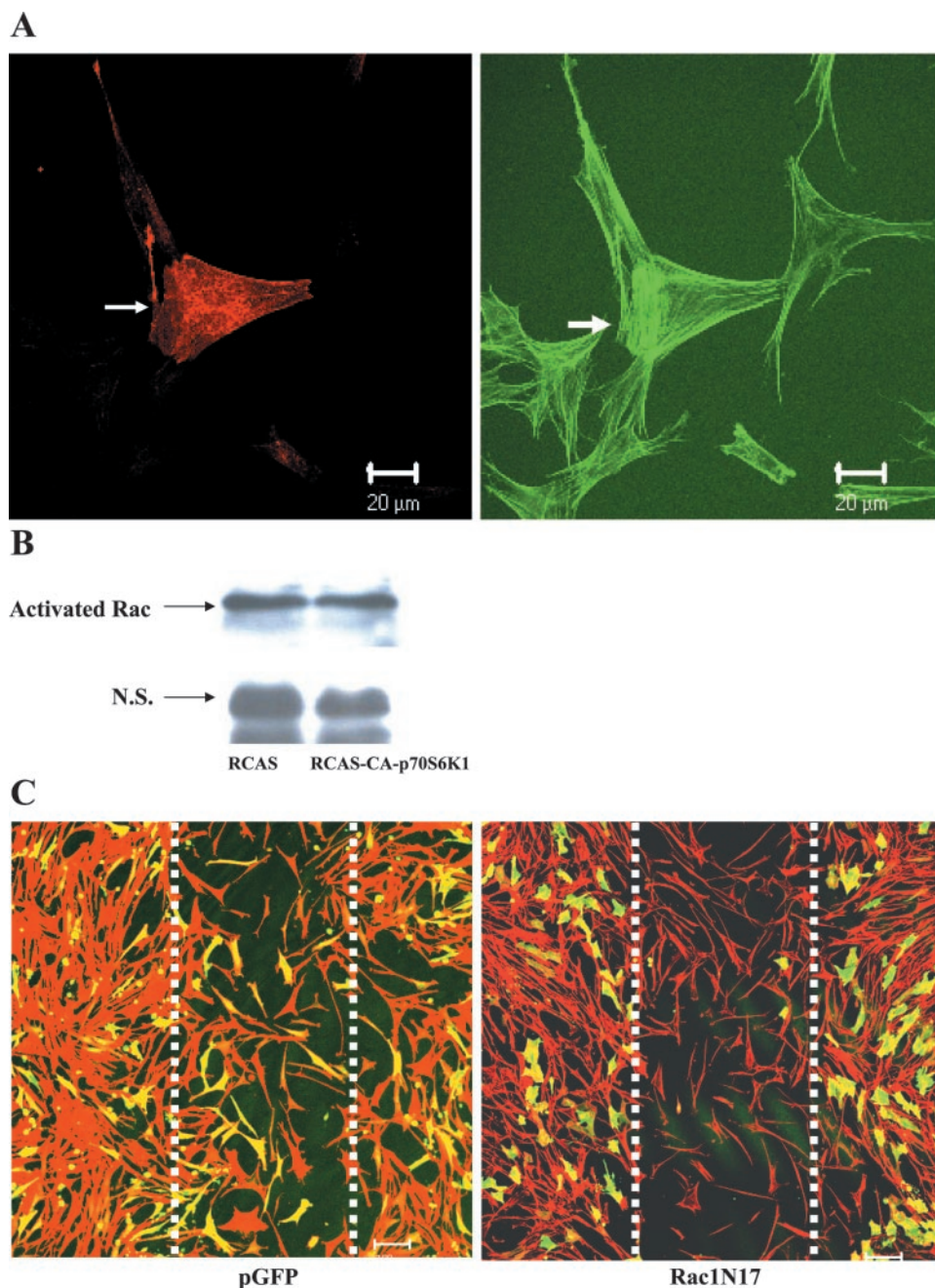


Fig. 7. Rac activity is required for p70S6K1-induced actin filament remodeling and cell migration. *A*: overexpression of a dominant negative construct of Rac1 inhibited p70S6K1-induced actin filament remodeling. CEF cells overexpressing the active form of p70S6K1 were cultured on coverslips and transiently transfected with a dominant negative form of Rac1, myc-tagged Rac1N17. After transfection for 48 h, cells were fixed and stained with FITC-phalloidin for detecting actin filaments and anti-myc antibody/TRITC-anti-mouse antibody for Rac1 expression. Arrows indicate a Rac1N17-transfected cell. *B*: p70S6K1 is unable to activate Rac. CEF cells overexpressing RCAS and RCAS-p70S6K1 were lysed, and the lysates were incubated with GST-PAK-CRIB to analyze the level of activated Rac. The GST-PAK-CRIB-bound Rac protein was resolved on 15% SDS-PAGE gel, followed by detection with an antibody against Rac. *C*: overexpression of the dominant negative form of Rac1 inhibited p70S6K1-induced cell migration. CEF cells expressing CA-p70S6K1 were cultured on coverslips and transiently transfected with either pGFP vector (*left*) or a dominant negative form of Rac1 (Rac1N17) (*right*). The cells were grown to 100% confluent monolayers and scratched to form a 100- μ m wound. After incubation for 24 h in 0.5% serum medium, the cells were fixed. The transfected cells were visualized as green (*left*: GFP-expressing cells; *right*: myc-tagged Rac1N17-expressing cells), and actin filaments were stained with TRITC-phalloidin as red. Lines indicate the wound.

activities would block CA-p70S6K1-induced cell migration, the wound-healing assays were performed. CEF cells expressing CA-p70S6K1 were transiently transfected with pGFP vector or a dominant negative Rac, pRK5mycRac1N17, and the transfected cells were examined for their ability to migrate toward the wound within 24 h. The GFP-expressing cells were able to migrate into the wound area within 24 h, whereas Rac1N17-expressing cells were unable to migrate into the wound area and remained within the edge of original monolayer cells. The results showed that expression of a dominant negative Rac1 reduced CA-p70S6K1-induced cell migration (Fig. 7C). Taken together, these results indicate that Rac activity is required for p70S6K1-induced actin filament remodeling and cell migration.

DISCUSSION

It has been demonstrated that the activity of PI3K is required for actin filament remodeling and cell migration in numerous cell signaling pathways. Much attention has been focused on identifying the downstream signaling proteins of PI3K in mediating the changes in cell morphology and motility. Although the phosphoinositides, the phospholipid products of phosphoinositide kinases, play a critical role in actin filaments remodeling and cell migration, no evidence has shown yet that the phospholipid products of PI3K, 3-phosphoinositides, have a direct role in the processes (36). Our studies demonstrated that overexpression of a constitutively active form of PI3K, v-P3k, is sufficient to induce the remodeling of actin filaments

to form lamellipodia and filopodia in CEF cells, consistent with the previous observations in endothelial cells (33).

We also demonstrated that overexpression of v-P3k is sufficient to induce cell migration. Furthermore, we found that v-P3k induces cell migration through the remodeling of actin filaments. Then, we further dissected the downstream signaling pathways in PI3K-induced actin filament remodeling and cell migration.

One of the major downstream targets of PI3K is the serine-threonine kinase Akt. Akt is an essential target of PI3K that transmits survival signals from growth factors (7). It has been found that v-P3k transmits the angiogenic and oncogenic signals through Akt (1, 19). Activated Akt has a variety of biological functions, including angiogenesis, glycogen synthesis, gene expression, inhibition of apoptosis, cell cycle arrest, endocytosis, vesicular trafficking, and cell transformation (5, 7). Role of actin filament remodeling in mediating cell migration by Akt was contrary. Akt was indicated to mediate the remodeling of actin filaments to mediate endothelial cell migration in response to VEGF (28). However, Akt was shown not to be involved in the cell migration through the remodeling of actin filaments of NIH3T3 cells in response to PDGF (17). The different results obtained in response to different growth factors could be due to the activation of different signaling pathways by the growth factors. In this study, we found that PI3K induced actin filament remodeling and increased cell migration. Several lines of evidence indicated that Akt is an essential downstream target of PI3K for the remodeling of actin filaments to increase cell migration. 1) PI3K induced the remodeling of actin filaments leading to an increase in cell migration, as well as the activation of Akt, which was completely abolished by a PI3K inhibitor, LY-294002. 2) Overexpression of the dominant negative forms of Akt abrogated PI3K-induced actin filament remodeling and cell migration. 3) The constitutively activated form of Akt was sufficient to induce actin filament remodeling and cell migration. 4) Activation of Akt was sufficient to induce actin filament remodeling and increase cell migration in the presence of PI3K inhibitor, LY-294002. 5) Inhibition of actin filaments with CD abolished both v-P3k- and Myr-Akt-induced cell migration. Thus Akt is a downstream signal protein stimulated by PI3K in regulating actin filament organization and cell migration. These results also indicate that the remodeling of actin filaments may play an important role in PI3K- and Akt-induced other cellular functions.

We further explored the downstream targets of Akt for inducing the remodeling of actin filaments. Inhibition of p70S6K1 activation by either rapamycin or a dominant negative form of p70S6K1 abolishes the effects of both PI3K and Akt on the remodeling of actin filaments and cell migration in CEF cells, demonstrating that p70S6K1 is required for PI3K and Akt-induced actin filament remodeling and cell migration. Our results are consistent with those observed in cell transformation, which demonstrated that cell transformation can be induced by either activated PI3K or activated Akt through the phosphorylation and activation of p70S6K1 (2). Our results are also consistent with those indicating that insulin, PDGF, and EGF activate p70S6K1 through the activation of PI3K and Akt (6). Our results were consistent with the recent observation that p70S6K1 plays a role in remodeling actin filaments in other cell systems (9). In thrombin signaling, the formation of stress

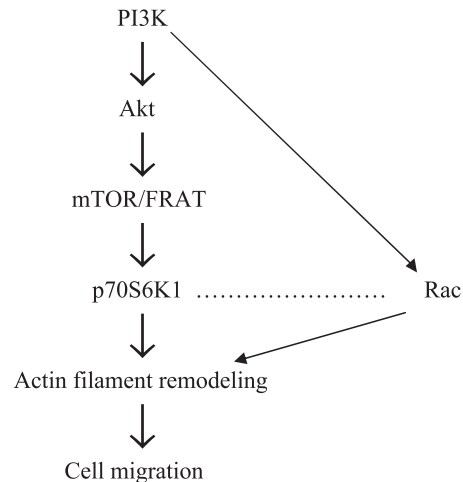


Fig. 8. Schematic representation of PI3K-induced actin filament remodeling and cell migration.

fibers upon thrombin stimulation is concomitant with both the activation of and the association of p70S6K1 with the stress fibers, and inhibition of p70S6K1 abolishes thrombin-induced stress fiber formation and prevents its localization on the stress fibers, indicating the functional roles of p70S6K1 in remodeling actin filaments in thrombin signaling (10). Berven and Crouch (4) speculated that p70S6K1 might have a functional role in cell migration via remodeling actin filaments based on their observations that p70S6K1 was localized at the areas of actin stress fibers and actin arc, an actin structure at the leading edge of cells, during cell movement. Our results directly demonstrate that overexpression of a constitutively active form of p70S6K1 is sufficient to induce actin filament remodeling to form lamellipodia and filopodia structures and to decrease actin stress fiber, leading to an increase in cell migration. In this study, we further examined the downstream signals of p70S6K1 in actin filaments remodeling and cell migration. Our results showed that the Rac activity was required for p70S6K1-mediated actin filament remodeling and cell migration (Fig. 7A). However, overexpression of an active form of p70S6K1 was unable to activate Rac protein, suggesting that Rac is regulated by another signaling pathway. Taken together, our report demonstrates that PI3K, Akt, or p70S6K1 alone is sufficient to induce the remodeling of actin filaments in CEF cells, which implies an increase of cell locomotility. The cell migration was assayed using serum as a chemoattractant. The result indicated that the remodeling of actin filaments led to an increase in cell migration, which is not due to the increase in cell proliferation during the assay (Fig. 6E). Akt is an essential target of PI3K in the induction of actin filament remodeling and cell migration, and Akt transmits the signal through the activation of p70S6K1. The integrity of Rac activities is required for p70S6K-mediated actin filament remodeling and cell migration. Therefore, we define a unique pathway that shows how PI3K induces the remodeling of actin filaments and increases cell migration; that is, PI3K → Akt → p70S6K1 → actin filament remodeling → cell migration (Fig. 8).

GRANTS

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