A Role for p21-Activated Kinase in Endothelial Cell Migration

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Abstract. The serine/threonine p21-activated kinase (PAK) is an effector for Rac and Cdc42, but its role in regulating cytoskeletal organization has been controversial. To address this issue, we investigated the role of PAK in migration of microvascular endothelial cells. We found that a dominant negative (DN) mutant of PAK significantly inhibited cell migration and increased stress fibers and focal adhesions. The DN effect mapped to the most NH$_2$-terminal proline-rich SH3-binding sequence. Observation of a green fluorescent protein-tagged α-actinin construct in living cells revealed that the DN construct had no effect on membrane ruffling, but dramatically inhibited stress fiber and focal contact motility and turnover. Constitutively active PAK inhibited migration equally well and also increased stress fibers and focal adhesions, but had a somewhat weaker effect on their dynamics. In contrast to their similar effects on motility, DN PAK decreased cell contractility, whereas active PAK increased contractility. Active PAK also increased myosin light chain (MLC) phosphorylation, as indicated by staining with an antibody to phosphorylated MLC, whereas DN PAK had little effect, despite the increase in actin stress fibers. These results demonstrate that although PAK is not required for extension of lamellipodia, it has substantial effects on cell adhesion and contraction. These data suggest a model in which PAK plays a role coordinating the formation of new adhesions at the leading edge with contraction and detachment at the trailing edge.

Key words: Rac • Cdc42 • cell motility • cytoskeleton • contractility

ENDOTHELIAL cell (EC)[1] migration is crucial in vasculogenesis, angiogenesis, and the repair of injuries along the endothelium. For cells to migrate, they must adhere and de-adhere to the substratum in a coordinated and polarized fashion. This process involves extension of filopodia and lamellipodia at the leading edge, formation of new adhesions in this region, and detachment of adhesions at the trailing edge of the cell (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Formation of lamellipodia and filopodia are regulated by the small GTPases, Rac and Cdc42, respectively (reviewed in Hall, 1998). These GTPases also promote formation of small adhesive structures called focal complexes, which are similar to the focal adhesions induced by Rho, but are smaller and appear only at the cell periphery (Nobes and

Hall, 1995). Extension of lamellipodia and filopodia and formation of new adhesions are key elements in cell migration, thus, these effects on the cytoskeleton reflect the fact that Rac and Cdc42 are important regulators of cell motility (Allen et al., 1998; Hall, 1998; Nobes and Hall, 1999).

Numerous effectors have been identified for Rac and Cdc42, however, which of these mediate effects of GTPases on the cytoskeleton or cell motility is not clear. The best characterized effector is p21-activated kinase (PAK; Manser et al., 1994; Lim et al., 1996). PAKs comprise a family of homologous serine/threonine kinases (∼62–68 kD), all of which are activated by binding to the GTP-bound form of Rac or Cdc42 (Manser et al., 1994; Knaus et al., 1995; Lim et al., 1996). There are at least three mammalian PAK isoforms that have been isolated, named PAK-1, PAK-2, and PAK-3 (Manser et al., 1994; Bagrodia et al., 1995; Martin et al., 1995). These proteins have highly conserved kinase and p21 binding domains (Lim et al., 1996), suggesting similar function and regulation. Both growth factors (Bokoch et al., 1996; Galisteo et al., 1996) and extracellular matrix proteins (Price et al., 1998) stimulate PAK activity. However, the role of PAK in regulating cytoskeletal organization downstream of Rac or Cdc42 has been controversial. On one hand, PAK overexpression

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1. *Abbreviations used in this paper:* AC, active CAAX tagged; EC, endothelial cell; DN, dominant negative; FN, fibronectin; GFP, green fluorescent protein; HMEC, human microvascular endothelial cells; JNK, Jun kinase; MLC, myosin light chain; PAK, p21-activated kinase; P-MLC, phosphorylated myosin light chain; WT, wild-type.

can induce changes in cytoskeletal organization, including formation of lamellipodia and turnover of actin stress fibers and focal adhesions (Manser et al., 1997; Sells et al., 1997; Zhao et al., 1998). In addition, PAK is recruited to membrane ruffles and focal complexes (Dharmawardhane et al., 1997; Manser et al., 1997). When overexpressed, PAK can trigger activation of Jun kinase (JNK) or the related p38 kinase (Bagrodia et al., 1995; Polverino et al., 1995; Zhang et al., 1995; Tang et al., 1997). PAK may also enhance activation of MAP kinase (Frost et al., 1997; Tang et al., 1997), which can promote cell migration (Klemke et al., 1997). However, activated mutants of Rac and Cdc42 that fail to bind or activate PAK still induce normal cytoskeletal structures and JNK activation (Lamarche et al., 1995; Westwick et al., 1997). Thus, the physiological role of PAK remains unclear.

There is more to cell migration than simply formation of extensions and adhesions. Cytoskeletal dynamics must also be properly organized and coordinated in time and space, which is an inherently complex process. Our approach to understanding the role of PAK in cytoskeletal organization was to study its role in migration, where effects might be evident. Both dominant negative (DN) and dominant active PAK mutants inhibit microvascular EC migration without affecting the formation of lamellipodia. The effects of these mutants on the rate of cell migration and cytoskeletal dynamics were characterized, and the sequence responsible for the DN effects mapped to a single proline-rich domain. Our data indicate that PAK is important for EC migration and suggest that it may be involved in coordinating the formation of new substrate adhesions at the front of the cell with contraction and detachment at the rear.

Materials and Methods

Cells

Human microvascular endothelial cells (HMEC-1) cells (Ades et al., 1992) were maintained and passaged in endothelial growth medium (Clonetics) supplemented with an additional 8% FBS (Gemini) for a final concentration of 10%. For some experiments, cells were transferred to endothelial basal medium supplemented with 0.2% FBS (Clonetics). Cells were maintained at 37°C in a humidified incubator containing 7% carbon dioxide.

DNA

cDNA expression plasmids containing full-length PAK1 and its various mutants in pCMV6M (CMV promoter, NH$_2$-terminal myc tag) have been described elsewhere (Sells et al., 1997; Daniels et al., 1998). Anti-PAK antiserum was raised as described (Knaus et al., 1995).

Pak Assay

Cells were extracted with buffer containing 1% NP-40 plus protease and phosphatase inhibitors, and PAK was immunoprecipitated using a polyclonal antiserum as described (Price et al., 1998). Kinase activity was determined using an in-gel kinase assay with myelin basic protein, and PAK protein levels were determined by Western blotting using the same antibody (Price et al., 1998).

Transfection of Cos-1 Cells

Cos-1 cells grown to 75% confluence on 10-cm tissue culture dishes were transiently transfected using the lipofectamine transfection protocol (GIBCO BRL) with a total of 7.5 μg of pCMV6M expression vectors con-
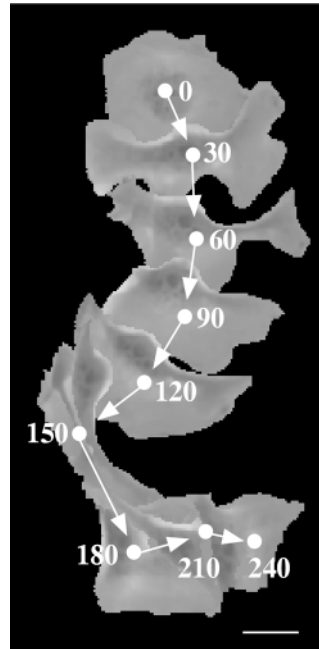

Figure 1. Tracking migration. Time-lapse phase-contrast images acquired at 30-min intervals of the same EC that was outlined and tracked using the Inovision software. The white circle defines the position of the centroid and the arrows the translocation of the centroid from each successive time point. Bar, 20 μm.

taining various myc-tagged constructs. The cells were allowed to express the protein for 40 h after transfection and were then washed in PBS and scraped into 250 μl of lysis buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl$_2$, 1 mM DTT, 5% glycerol, 1% NP-40) at 4°C. Lysates were passed four times through a 21 gauge needle and then clarified by centrifuging for 5 min at 7,000 rpm in a benchtop Eppendorf microfuge to remove unbroken cells and large cellular debris. Supernatants were decanted and 30 μl of each lysate was run on SDS-PAGE gels and transferred to nitrocellulose before probing for the expression of myc-tagged protein using an anti-myc antibody (9E10); the remainder was immediately frozen at −80°C until required.

Microinjection and Real-time Video Phase-contrast Microscopy

To assay migration, HMEC-1 cells were plated on coverslips coated with 2 μg/ml of fibronectin (FN; Sigma Chemical Co.). Dishes were prepared by cutting a hole in the bottom of the dish and attaching a coverslip to the outside of the dish with silicone grease. These were placed in an open chamber with atmospheric and temperature control (Schwartz, 1993) and
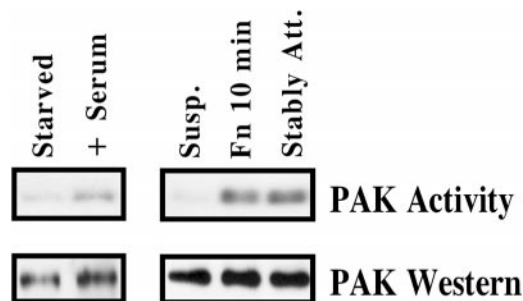

Figure 2. PAK activity assays. PAK was immunoprecipitated from lysates obtained from ECs that had been maintained in basal medium (Starved), stimulated with growth medium for 10 min (+ Serum), detached and kept in suspension in basal medium (Susp.), replated on FN for 10 min (Fn 10 min), or left stably attached in basal medium (Stably Att.). PAK kinase activity was assayed toward myelin basic protein. Top, PAK activity; bottom, Western blot of PAK protein in the immunoprecipitates.

viewed with a Nikon DiaPhot microscope equipped with a SenSys cooled CCD video camera linked to a Silicon Graphics workstation running the Inovision ISEE software program. HMEC-1 ECs were injected with cDNAs at 0.2 mg/ml as described (Meredith et al., 1995). Protein expression was detectable by immunofluorescence ~30 min after injection and cell migration was assessed by time-lapse imaging beginning 60 min after injection. At the end of the experiment, images of cells were outlined and the centroid (cell center) calculated. Displacement of the centroid was then used to determine movement over time (Fig. 1).

NIH 3T3 cells were plated on 50 μg/ml of FN in medium with 10% calf serum to match the conditions used by Sells et al. (1999). They were allowed to spread for 2 h before injection and migration was measured as described.

Fluorescence Microscopy

Cells were fixed for 10 min in 3% paraformaldehyde in PBS, permeabilized for 10 min with 0.01% Triton X-100/PBS, and rinsed twice with PBS. Coverslips were blocked by incubating for 40 min in 10% goat serum at
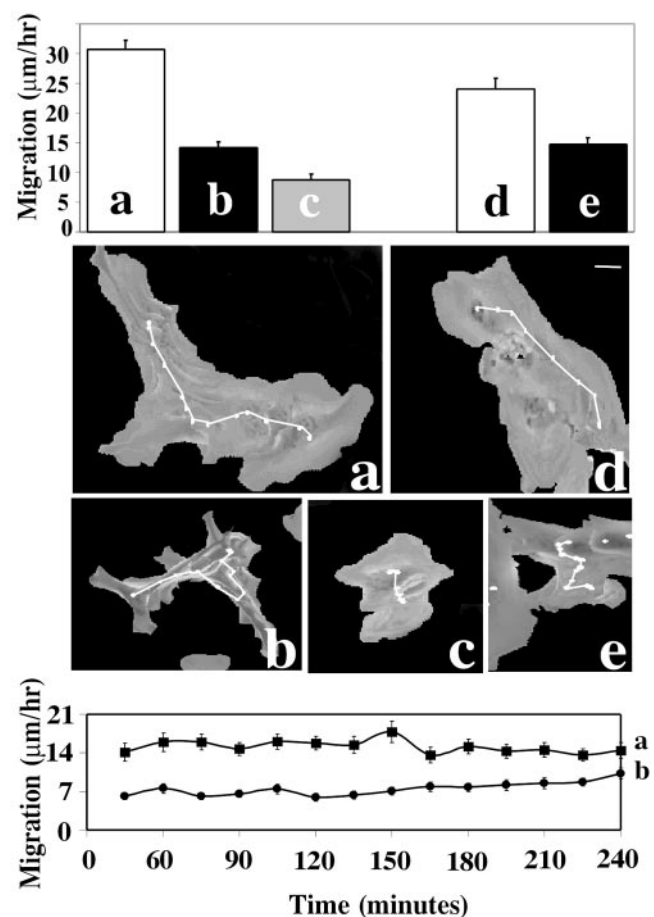
room temperature, then stained for 60 min in a 1:10 dilution of rhodamine-phalloidin (ICN Immunobiologicals). Coverslips were then washed twice in PBS before being incubated with a 1:100 dilution of anti-vinculin (Sigma Chemical Co.) antibody (or anti-HA, or myc epitope antibodies [Berkeley Antibody Co.], or a polyclonal anti-phosphorylated myosin light chain [anti–P-MLC; gift of Dr. F. Matsumura, Rutgers University; Matsumura et al., 1998] in other experiments), followed by washing with PBS. Coverslips were incubated in a 1:100 dilution of CY5-conjugated goat anti–mouse F(ab′)2 fragment (Sigma Chemical Co.) or CY5 goat anti–rabbit (H & L; Sigma Chemical Co.) for 60 min, washed twice in PBS, and mounted in Immunofluore mounting medium (ICN Immunobiologicals). Slides were viewed using a BioRad 1024 MRC scanning confocal microscope.

Assay for Phosphorylated Myosin Light Chain

ECs, which were injected with DN, active CAAX tagged (AC), or wild-type (WT) PAK were fixed and stained for actin and P-MLC as described above. Images were then processed using a Silicon Graphics workstation running the Inovision ISEE software program. Cells were outlined and total fluorescence staining intensity determined by determining the sum of the pixel intensities per cell. Background staining in cell-free areas was negligible. At least 20 cells were analyzed from four experiments for each value.

Analysis of GFP–α-Actinin

HMEC-1 ECs were coinjected with cDNAs at 0.2 mg/ml of various PAK constructs, together with 0.2 mg/ml of cDNA coding for green fluorescent protein (GFP)–α-actinin fusion protein. Starting at ~6 h after injection, at which time the fluorescence signal was sufficiently intense, cells were monitored for up to 16 h after injection under fluorescence optics on the inverted Nikon microscope using a 20× oil immersion lens. Images of cells were collated and viewed by time-lapse using a Silicon Graphics workstation running the Inovision ISEE software program.

Figure 3. Stimulation of migration by growth factors. Top, Rate of migration of uninjected ECs after plating on 2 μg/ml FN in the presence of growth medium (a) or basal medium (b), or 24 h after plating on 2 μg/ml FN in basal medium (c). Migration of cells injected with GFP alone was also determined after plating cells on 2 μg/ml FN in the presence of growth medium (d) and basal medium (e). Middle, Composite time-lapse phase-contrast images of cells, shown at 30-min intervals, in the conditions outlined above (labeled a–e, respectively). Bar, 20 μm. Bottom, Rate of migration as a function of time after plating. Uninjected ECs were monitored after plating on 2 μg/ml FN in the presence of growth medium (a) and basal medium (b). Rate of migration was determined during each 30-min time interval.
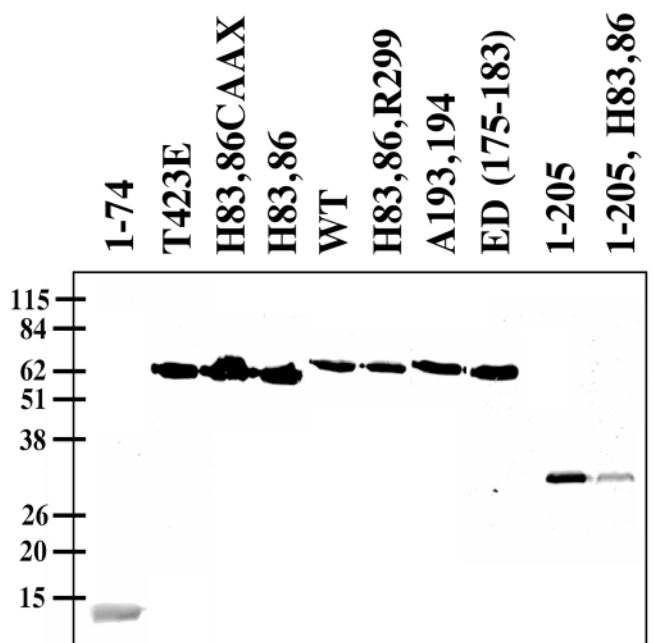

Figure 4. Expression of PAK constructs. A Western blot showing expression of PAK constructs in COS-1 cells. Cells were transiently transfected using lipofectamine for 40 h with various constructs, lysed in PBS with 1% NP-40, and equal amounts of lysate were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with myc antibody to recognize the tagged proteins (as described in Materials and Methods). Expressed proteins migrated at the expected molecular weights.

Silicone Rubber Substrates

To visualize cell contractility, flexible rubber substrates were generated by a protocol described in Chrzanowska-Wodnicka and Burridge (1996). In brief, silicone rubber (dimethyl polysiloxane), viscosity 10,000 centistokes (Dow Corning Co.), was coated onto the coverslip at the bottom of 35-mm tissue culture dishes and allowed to spread for 2 h. The silicone was then coated with a thin layer of gold-palladium using a Hummer VI sputter coater. The UV glow discharge during the coating process polymerized the silicone rubber and the gold-palladium coating decreased the hydrophobic nature of the rubber surface; the surface was additionally coated with 2 μg/ml of FN. ECs were plated on the rubber substrate and allowed to adhere for ∼30 min, then, only cells that had wrinkled the substrate beneath were coinjected with the various PAK constructs and GFP, and incubated for 4 h. Expressing cells were recognized by GFP fluorescence and the ability of the cells to create wrinkles in the substratum was scored by phase-contrast microscopy.

Quantification and Statistical Analysis

Statistical analysis was performed using Microsoft Excel statistics software. The displacement of the centroid over time and thus the rate of cell migration was obtained with the Inovision ISEE software program from images obtained using a 10× objective. Approximately 25 cells were injected per experiment, with at least three independent experiments for each PAK construct. Results were expressed as mean ± SEM. The t test was used to determine whether the observed differences were statistically significant ($P = 0.05$).

Results

PAK Activity in HMEC-1 Cells

Previous work in fibroblasts has shown that PAK1 kinase activity is stimulated by both integrin-mediated adhesion to extracellular matrix proteins and by serum or soluble growth factors (Bokoch et al., 1996; Galisteo et al., 1996; Price et al., 1998). Therefore, we tested whether PAK1 in HMEC cells behaves similarly. Serum starved cells were detached and either replated on FN or kept in suspension. Additionally, adherent starved cells were stimulated with growth medium containing serum and basic fibroblast growth factor. PAK1 was immunoprecipitated from cell lysates and its activity toward myelin basic protein assayed. Fig. 2 (right) shows that PAK activity in suspended cells was nearly undetectable, and was stimulated by about tenfold upon reattachment to FN. PAK activity was maximal by ten minutes after replating and was sustained, as it was similar to the level in serum starved stably adherent cells. A separate experiment (Fig. 2, left) shows that the level of PAK activity in stably adherent cells in basal medium was further increased by addition of growth medium
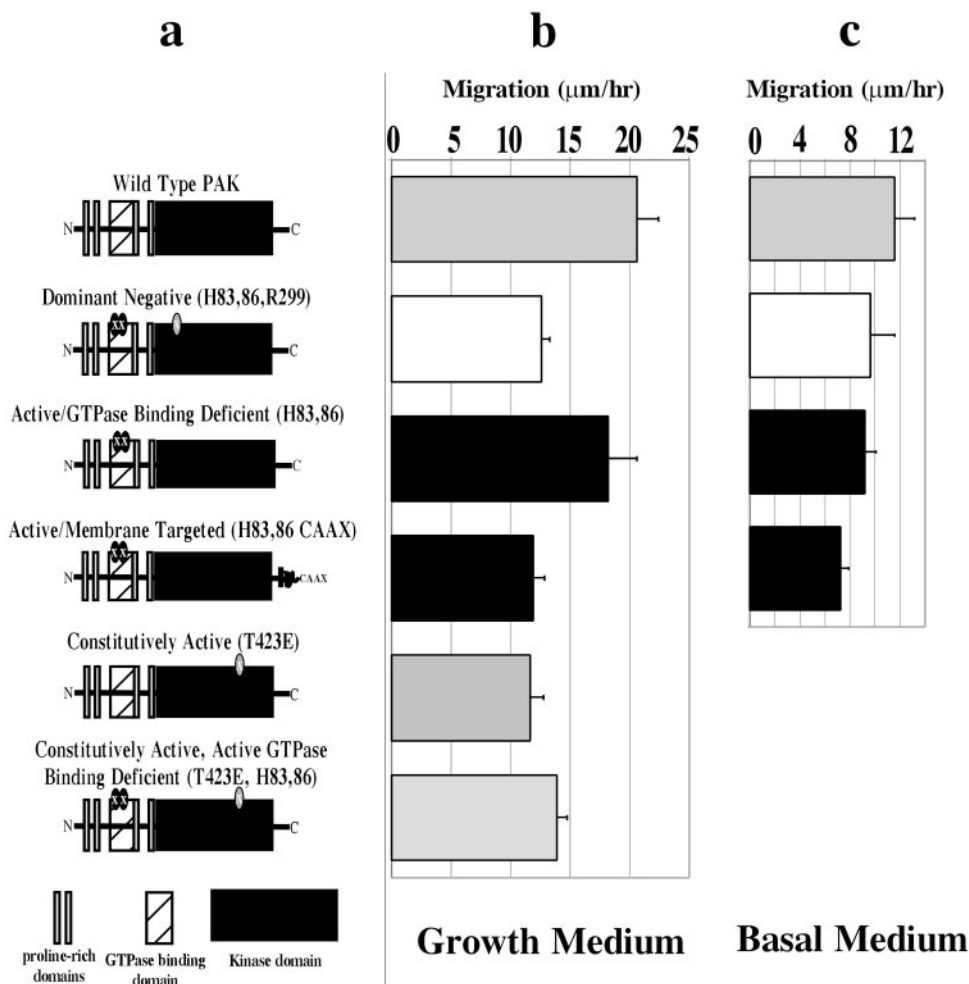

Figure 5. Effects of PAK constructs on cell migration. a, Diagrammatic representations of PAK constructs. Critical domains of the PAK constructs are indicated at the bottom of the figure and labeled proline-rich, GTPase binding domain, and kinase domain, accordingly. b, Rates of migration (mean ± SD) of ECs plated on 2 μg/ml of FN in growth medium after injection with the constructs immediately to the left in a. c, Rates of migration (mean ± SD) of ECs plated on 2 μg/ml of FN basal medium after injection with the constructs in the left in a.

by ~1.7-fold. These data show that both adhesion and growth factors contribute to PAK activity in HMECs, confirming that PAK in ECs behaves similarly to PAK in fibroblastic cells.

Migration of HMEC-1 Cells

Initial measurements of HMEC-1 migration showed that cells plated the day before the experiment in basal medium migrated at a very low rate (Fig. 3, top). Indeed, much of the apparent migration was due to changes in the location of the centroid due to extension and retraction of processes, rather than true displacement of the cell body. Cells that were plated on coverslips coated with 2 µg/ml FN spread within 15 min and, after one hour, migrated at a slightly but consistently faster rate. This rate persisted for at least four hours (Fig. 3, bottom) and additional experiments showed no change for up to eight hours (not shown). These results suggest that the increase was due to migration on the FN coating as opposed to the matrix synthesized by the cells, and did not represent transient stimulation due to replating, per se. The rate of migration of cells plated on FN in growth medium was ~2.5-fold higher than in basal medium. This rate was also maintained for at least four hours after plating (Fig 3, bottom), and additional experiments showed no change for up to eight hours (not shown). Examples of cell displacement for each condition are shown to illustrate typical patterns of cell movement (Fig. 3, middle).

To analyze the effects of PAK mutants, we chose to express these constructs by microinjection, since this method gives efficient expression even in ECs that are difficult to transfect. This approach also avoids potential longer term effects of PAK mutants on gene expression. To confirm the size of the expressed proteins, all of the constructs used in these and the following experiments were overexpressed in COS-1 cells and analyzed by Western blotting. The constructs were expressed at similar levels and yielded proteins of the expected sizes (Fig. 4).

Microinjection of cells with a control GFP construct slowed the rate of movement of the cells in growth medium by ~30%, though it had little effect on cells in basal medium (Fig 3). This modest inhibition presumably indicates the effect of microinjection alone and is consistent with results obtained in other studies (Allen et al., 1998). Coexpression of WT PAK with the GFP did not significantly alter cell migration compared with GFP alone (Figs. 3 and 5). Injected cells that expressed the heterologous proteins could be identified either by staining with antimyc to detect the tagged PAK or by fluorescence of the GFP. Since these two always correlated (not shown), we routinely relied upon GFP fluorescence to detect expressing cells in subsequent experiments. All of the PAK constructs gave similar staining intensity with anti-myc antibody (not shown), indicating similar expression levels in HMEC-1 cells.
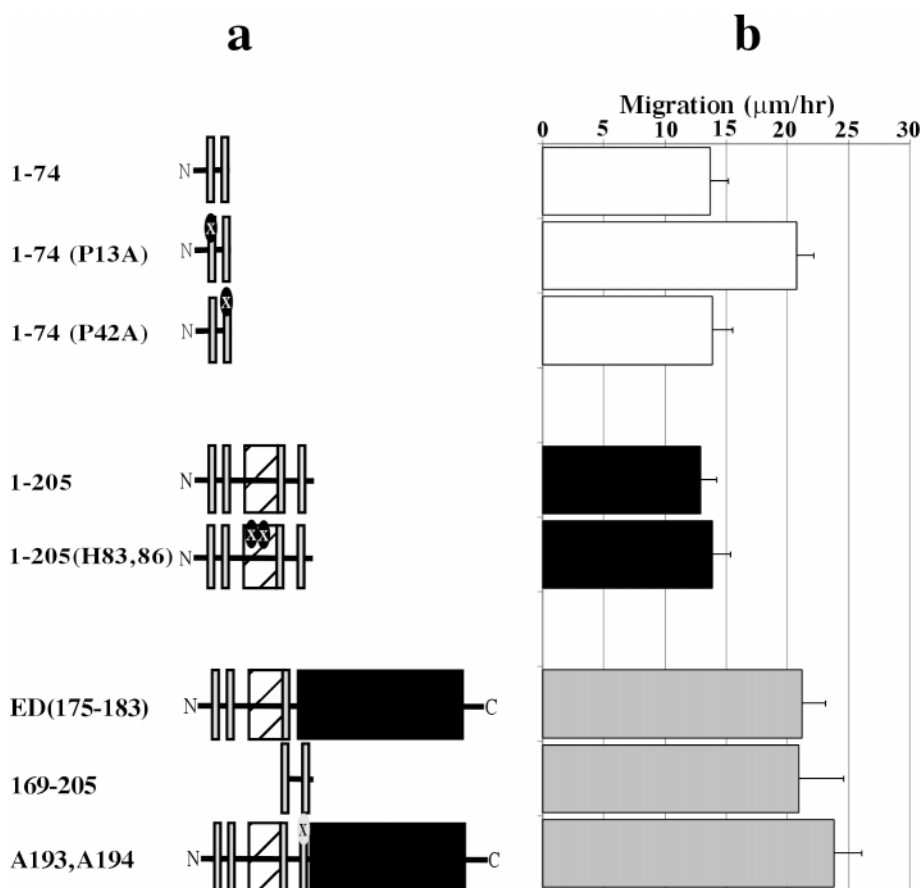

Figure 6. Mapping the DN effect. a, Diagrammatic representations of PAK constructs. b, Rate of migration of ECs plated on 2 µg/ml of FN in the presence of growth medium after injection with the constructs shown immediately to the left in a. Values are mean ± SD.

Effects of Dominant Negative and Constitutively Active PAKs

HMEC-1 cells were coinjected with GFP plus a DN PAK containing mutations in both the kinase domain (R299) and the GTPase binding domain (H83L, H86L). This construct lacks kinase activity and also fails to bind Rac or Cdc42, therefore, it does not sequester the endogenous GTPases to inhibit their interactions with other effectors. Previous studies showed that this DN PAK interferes with function of endogenous PAK (Sells et al., 1997; Tang et al., 1997), presumably by associating with substrates or other PAK-interacting proteins. Expression of this construct decreased the rate of migration in growth factor–stimulated cells to the level in unstimulated cells ($P < 0.001$; Fig. 5). A slight decrease was also noted in unstimulated cells, but

this effect was not statistically significant ($P = 0.45$). This result suggests that PAK may be involved in growth factor–stimulated EC migration.

To further investigate the role of PAK in migration, four constitutively active mutants of PAK were also examined. T423E PAK and H83L,H86L PAK are activated variants; the T423E mutant shows significantly higher kinase activity in vitro (Sells et al., 1997), whereas the H83L,H86L mutant is also deficient in binding to Rac and Cdc42, and therefore should not inhibit other pathways downstream of the GTPases. Additionally, we tested a combined mutant, T423E,H83L,H86L PAK, which is strongly activated and deficient in binding the GTPases, and a membrane-targeted active variant of this latter mutant, H83L,H86L,AC PAK, which is activated, deficient in GTP-
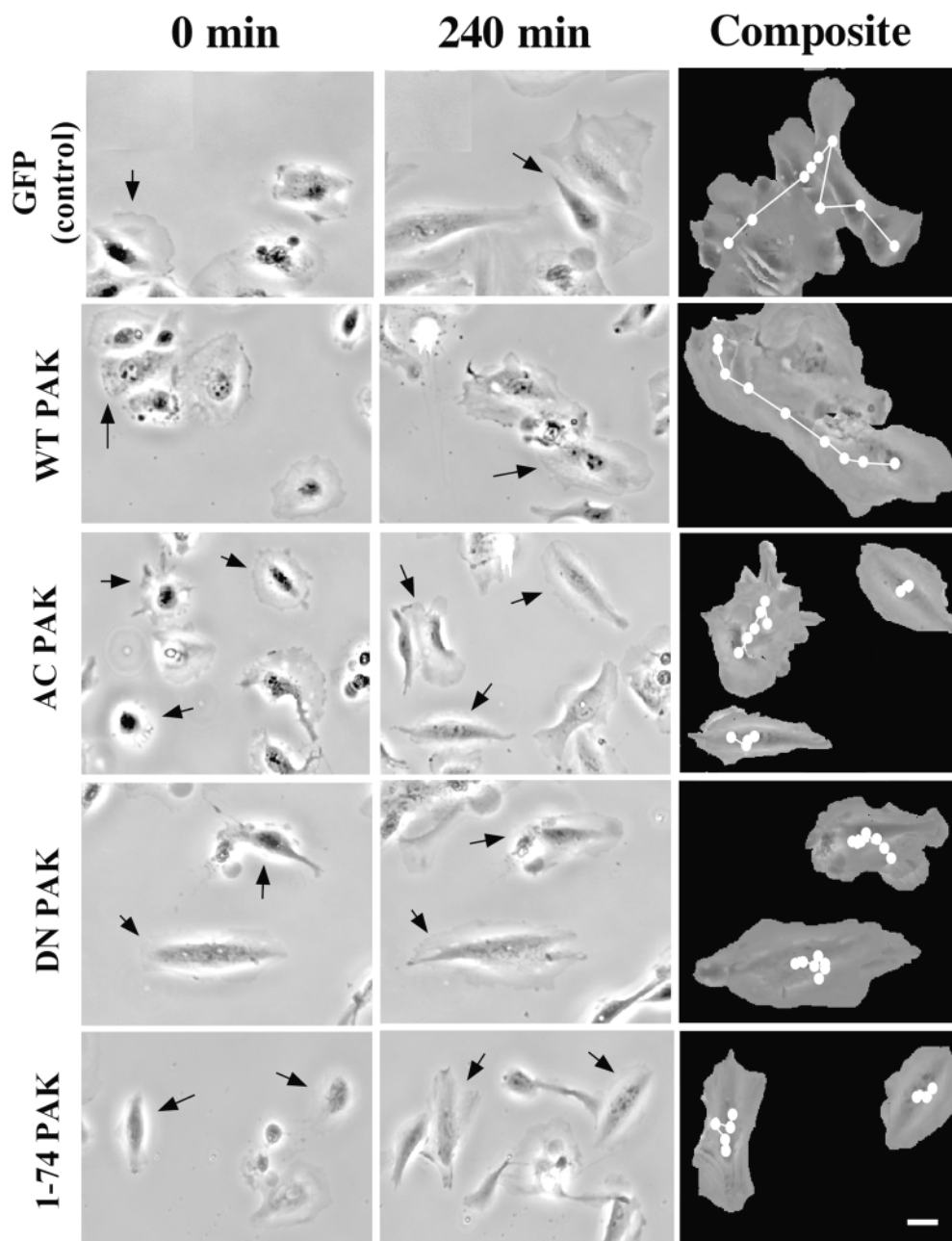

Figure 7. Morphology of migrating cells. Time-lapse series of phase-contrast micrographs showing 0 and 240 min, as indicated. The final panel outlines the composite phase-contrast images over the 4-h period. Cells were injected with GFP only (control), WT PAK, AC PAK, DN PAK, or the 1-74 PAK fragment. Expressing cells are indicated by arrows in each panel. Micrographs demonstrate similar levels of ruffling and lamellipodia, despite lack of migration in AC, DN, and 1-74 PAK.

ase binding, and membrane targeted due to the presence of a COOH-terminal isoprenylation sequence (Daniels et al., 1998). Cell migration was assayed both in basal medium and growth medium. While the weakly activated 83,86 mutant had only a small effect that did not reach statistical significance ($P > 0.05$), the strongly activated T423E mutant and the membrane targeted 83,86 mutant both significantly inhibited cell movement ($P < 0.001$) to an extent that was similar to DN PAK (Fig. 5). The combined mutant T423E,H83L,H86L also significantly inhibited migration, indicating that effects of T423E were not due to sequestering the GTPases. Notably, some inhibition by the membrane targeted construct also occurred in cells without growth factors ($P < 0.05$); the 83,86 mutant that did not target to membranes decreased migration of starved cells slightly, but the effect did not reach statistical significance ($P = 0.22$). One might have expected that expression of active PAK constructs would stimulate migration in the starved cells, however, the effects were, if anything, weakly inhibitory. These results argue that proper regulation of PAK is required for cell migration. Thus, either unregulated increases or decreases in PAK activity inhibit motility.

Mapping Sequences that Mediate the Dominant Negative Effect of PAK

To further investigate interactions of PAK that were important for inhibiting HMEC-1 cell migration, we tested additional constructs designed to probe smaller regions of the molecule (Figs. 5 and 6, left). These constructs allowed us to investigate the relative contributions of different domains. As shown in Fig. 6, a full-length construct in which the third proline-rich domain, which is known to bind the Rac/Cdc42 nucleotide exchange factor PIX (Manser et al., 1998), was mutated to block binding to SH3 domains (A193,A194) and had no effect on migration. Mutation of the ED (glutamic and aspartic acid-rich) domain also had no effect in the context of full-length PAK. Expression of the NH$_2$-terminal regulatory domain of PAK (1-205) inhibited migration, as well as the full-length DN construct, indicating that important inhibitory sequences resided in the NH$_2$-terminal half of PAK. A 1-205 (H83L,H86L) construct that lacked GTPase binding inhibited migration equally well, indicating that this effect did not require blocking Rac or Cdc42 function. The extreme NH$_2$ terminus contains 2 putative SH3 domains (Bokoch et al., 1996; Galisteo et al., 1996). Expression of the first 74 residues that encompass these sites decreased migration as efficiently as full-length DN PAK, indicating that this region was sufficient to inhibit migration. Mutation of the first proline rich sequence (P13A) prevented the decrease in migration rate, whereas mutation of the second proline-rich sequence (P42A) had no effect. These results indicate that the first proline rich sequence of PAK is critical to its dominant effects on EC migration.

Effects of PAK on Cell Morphology and Cytoskeletal Organization

Since HMEC-1 cells were studied by time-lapse microscopy, information about cell morphology was also readily available. Similar to HMEC-1 cells injected with GFP

alone or WT PAK plus GFP, those injected with DN PAK continued to extend lamellipodia and ruffle. They tended to spread more extensively than control cells, especially in basal medium, despite their low rate of translocation (Fig. 7). Cells with DN PAK also typically had circular or oval shapes with fewer extensions than either WT or AC PAK (Figs. 7 and 8). Interestingly, cells injected with active, membrane-targeted AC PAK showed lamellipodia and ruffles similar to control cells, but translocated very little in comparison to control cells (Fig. 6). The T423E mutant
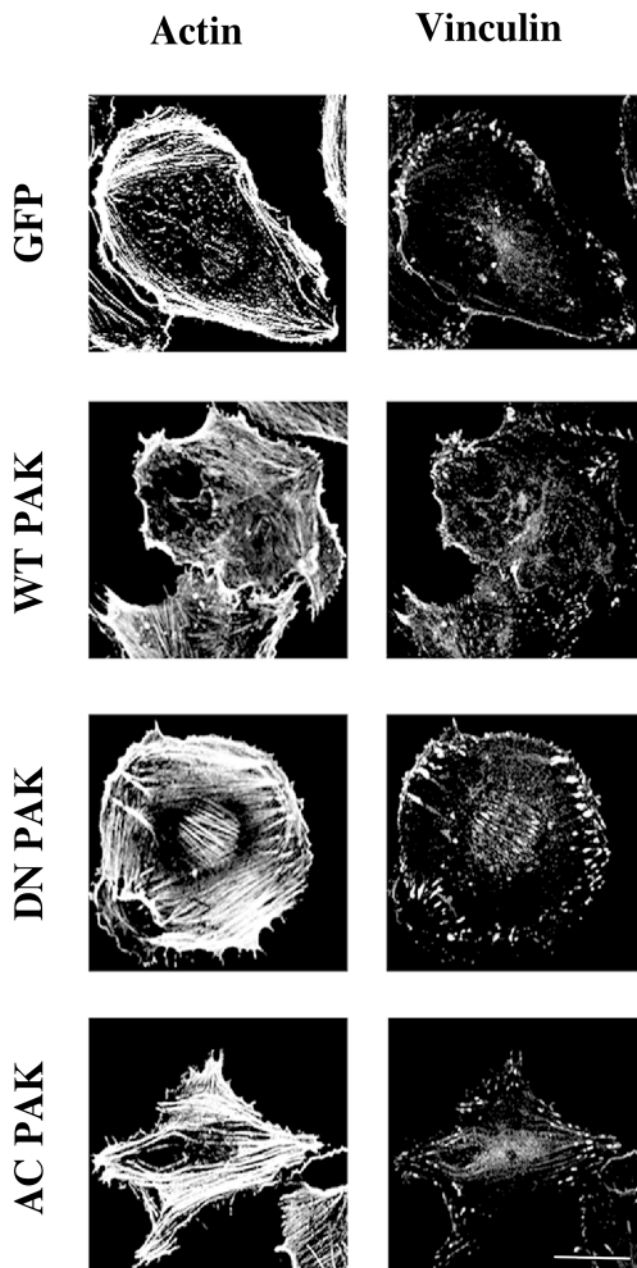

Figure 8. Cytoskeletal structure. Fluorescence micrographs of ECs stained for actin and vinculin after injection with GFP alone, WT PAK, DN PAK, or AC PAK. Cells injected with DN and AC PAK show an increase in stress fibers and vinculin-containing focal contacts in comparison to GFP alone and WT PAK-injected ECs. Bar, 20 μm.
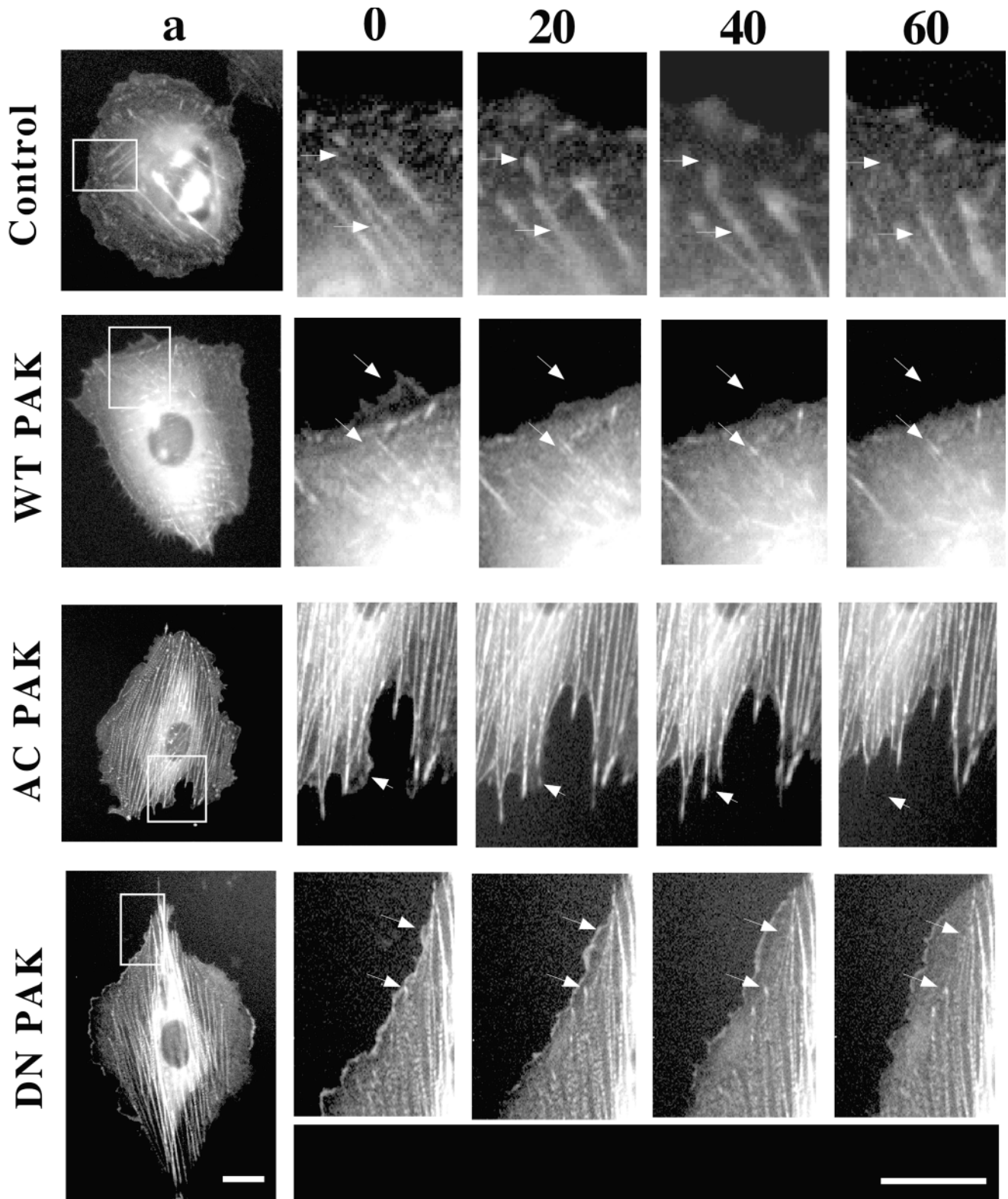
Figure 9. Cytoskeletal dynamics. Fluorescence images taken at 20-min intervals of ECs injected with GFP–α-actinin alone (Control) or coinjected with GFP–α-actinin plus WT PAK, AC PAK, or DN PAK. The first image in each set shows the entire cell (a). The next four images magnify the region within the box in a. The arrows in the magnified inserts for control and WT PAK show focal adhesions that disappear or grow and change shape. Bars, 20 μm. For AC PAK, the arrows identify an area where focal adhesions disappear and the cell retracts. For DN PAK, the upper arrow indicates a growing ruffle whereas the one below shows a stationary focal adhesion. Control and WT PAK-injected cells appear dynamic, whereas focal adhesions and stress fibers in cells injected with DN PAK are static. Cells injected with AC PAK are intermediate.

behaved similarly to the AC PAK (not shown). Because the activated constructs all gave similar results, the AC PAK construct was chosen for further studies, as it has strong effects, but avoids possible complications due to binding Rac or Cdc42.

To test effects of PAK on actin organization and focal contacts, injected cells were stained for actin and vinculin. As shown in Fig. 8, HMEC-1 cells expressing GFP alone or with WT PAK generally had modest numbers of stress fibers and focal adhesions. These cells were indistinguishable from uninjected cells (not shown). Cells expressing DN PAK showed increased stress fibers and larger focal adhesions. Similarly, expression of AC PAK also increased stress fibers and focal adhesions.

Effects of PAK on Cytoskeletal Dynamics

The results from the previous experiments are ostensibly paradoxical, since either inhibiting or activating PAK gave superficially similar effects (though differences in shape were noted). To gain further insight into the effects of PAK on the cytoskeleton, cells were injected with an expression plasmid coding for GFP-conjugated α-actinin, which labels both stress fibers and focal adhesions, and therefore permits their visualization in living cells (Fig. 9). The GFP–α-actinin was expressed alone, with WT PAK, or with other PAK constructs, and cells viewed using time-lapse fluorescence microscopy. Similar to results from the static images described above, both DN and AC PAK induced an increase in stress fibers and focal adhesions. Interestingly, in cells expressing GFP only or GFP and WT PAK, most stress fibers and focal adhesions were relatively stable, appearing or disappearing relatively slowly. However, once formed, these structures showed surprising flexibility. They changed positions extensively, with stress fibers lengthening, shortening, bending, and moving relative to the substrate and each other as the cells changed shape (Fig. 9, insets). By contrast, the α-actinin–containing structures in cells expressing DN PAK were almost completely static (Fig. 9). Whereas extensive lamellipodia and

ruffling was observed around the periphery of these cells, the inner network of actin cables and adhesions appeared virtually fixed in place. In addition, lamellipodia failed to show any polarity and were observed around the entire cell periphery.

HMEC-1 cells injected with AC PAK appeared intermediate between the DN and WT constructs. These cells were less dynamic than control cells, but more dynamic than those expressing DN PAK. Formation of new adhesions, retraction of areas of the cell edge and bending, shortening, and lengthening of actin cables still occurred, although less rapidly than in control cells. Substantial lamellipodia and ruffling were also observed as in cells expressing GFP alone and WT PAK. These results raise the possibility that DN and active PAK may in fact not have identical effects on the cytoskeleton.

Effect of PAK on Contractility

Cell migration requires that new adhesions form at the leading edge and that old adhesions detach at the rear of the cell. Detachment may involve both contraction and diminished adhesion strength, depending on the cell type (Hendey et al., 1992; Palacek et al., 1998). One hypothesis that could explain the apparently paradoxical effect of PAK constructs on migration and focal adhesion formation is that it plays a role in coordinating adhesion formation at the leading edge with contraction and detachment at the trailing edge (these ideas are explained more fully in the Discussion). To test the involvement of PAK in contraction, cells were plated on silicone rubber membranes that can be deformed by cell-generated tension (Harris et al., 1980; Chrzanowska-Wodnicka and Burridge, 1996). HMEC-1 cells plated on this deformable surface create wrinkles that are easily observed by phase-contrast microscopy. Expression of GFP alone or GFP plus WT PAK had little effect on these wrinkles. Cells injected with the AC PAK construct maintained or increased wrinkling of the substrate. This increase was difficult to quantitate, however, an increase in wrinkle depth or intensity was
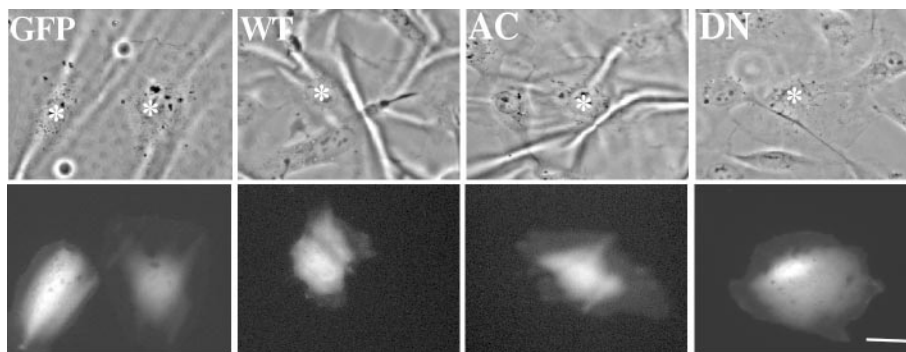

Protein Expressed	Contractile Cells	%
(a) GFP	54 out of 68	79
(b) GFP + WT PAK	48 out of 60	80
(c) GFP + AC PAK	44 out of 54	81
(d) GFP + DN PAK	18 out of 56	32

Figure 10. Cell tension on silicone rubber substrata. Phase-contrast images and corresponding fluorescence micrographs of ECs plated on 2 μg/ml FN coated onto flexible rubber substrates 4 h after injection with GFP alone, WT PAK, AC PAK, and DN PAK. Injected cells are labeled with an asterisk (*) in each phase-contrast panel. Bar, 20 μm. The chart at the bottom shows the number of ECs that induced wrinkling of the substrate, and the corresponding percentages accumulated from five experiments.

consistently observed (Fig. 10). The DN PAK, by contrast, caused a substantial decrease in wrinkling of the rubber substrate (Fig. 10). Quantification of these results showed that after injection with GFP, 79% of cells still wrinkled the rubber substrate. 80 and 81% of cells expressing WT PAK and AC PAK, respectively, also produced wrinkles. However, only 30% of cells expressing DN PAK induced wrinkles (Fig. 10). Addition of cytochalasin D at 1 μg/ml completely abolished all wrinkles (not shown), demonstrating that wrinkling required an intact actin cytoskeleton. These results show that PAK is required for contractility in HMEC-1 cells.

Effect of PAK on Myosin Light Chain Phosphorylation

Effects of PAK mutants on contractility and stress fiber formation suggest that myosin phosphorylation might be involved. Recent reports in different systems have been contradictory, however, since an active PAK mutant decreased phosphorylation of MLC in baby hamster kidney and HeLa cells (Sanders et al., 1999), but increased MLC phosphorylation in 3T3 cells (Sells et al., 1999). The microinjection strategy employed in our studies precludes biochemical assays of myosin phosphorylation, hence we resorted to staining with an antibody developed by Matsumura and colleagues that specifically recognizes phosphorylated MLC (Matsumura et al., 1998). Staining intensity was then quantified. These experiments revealed an increase in staining with anti–P-MLC in cells expressing the active PAK mutant, indicating an increase in MLC phosphorylation ($P < 0.00005$, relative to cells expressing WT PAK; Fig. 11). By contrast, the DN PAK construct caused only a slight and statistically insignificant change in staining with anti–P-MLC, even though it induced an increase in actin stress fibers.

Cell-Type Specificity

Other groups have reported that expression of the H83L,H86L–activated PAK in fibroblasts decreased actin stress fibers and focal adhesions (Manser et al., 1997; Sells et al., 1997). Additionally, stable expression of that construct in NIH 3T3 cells increased migration (Sells et al., 1999). To determine whether these discrepancies were due to distinct cell types or might be explained by different methods or cell lines used by different laboratories, we tested effects of the same construct in 3T3 cells by microinjection. We observed that microinjection of NIH 3T3 cells with cDNA for the 83,86 active PAK mutant triggered formation of lamellipodia and loss of actin stress fibers as described in the above reports (Fig. 12). When cell migration was assayed under the conditions employed by Sells et al. (1999), cells microinjected with vector coding for the active PAK construct moved at a rate of 15.7 ± 1.4 μm/h compared with 9.4 ± 1.3 for GFP vector only (a 70% increase, statistically significant $P < 0.05$). This result is consistent with the data obtained by Sells et al. (1999) and suggests that the observed differences are most likely due to cell-type specificity rather than different methods of expression. In light of these results on myosin phosphorylation, the data indicate that the cell-type–specific effects of PAK cannot be explained by differences in MLCK activ-

ity, suggesting the existence of a second pathway by which PAK modulates cytoskeletal dynamics.

Discussion

Our results show that expression of either inhibitory or activated PAK in HMEC-1 cells decreases growth factor–
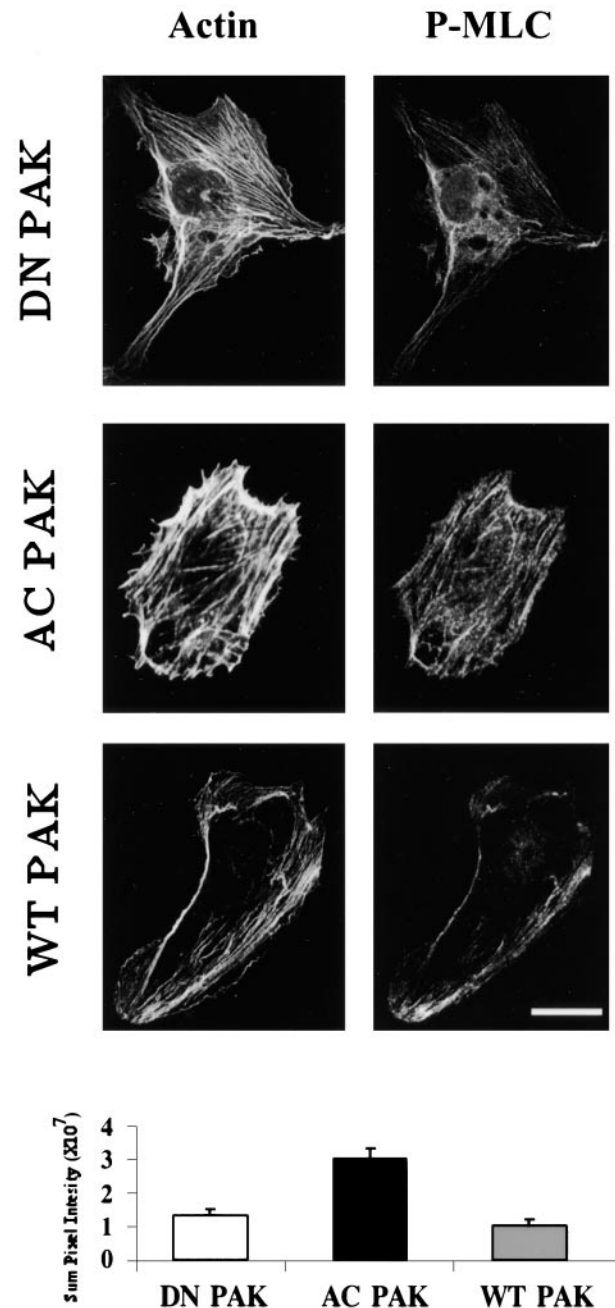

Figure 11. P-MLC staining fluorescence micrographs of ECs stained for actin and P-MLC after injection with WT PAK, DN PAK, or AC PAK. Cells injected with AC PAK show an increase in MLC staining in comparison to both DN and WT PAK-injected ECs. Bar, 20 μm. Lower panel indicates quantitation of fluorescence intensity of P-MLC stained cells. Values represent mean ± SEM from 15–20 cells. Similar results were observed across four independent experiments.

stimulated migration to the level in unstimulated cells. The effect of the DN construct was mapped to a single short proline-rich sequence that is known to bind the SH3 domain of the adapter protein Nck (Bokoch et al., 1996; Galisteo et al., 1996). Whether Nck itself or another SH3-containing protein is involved in this effect on migration is currently unknown. The DN PAK construct also induced an increase in focal adhesions and stress fibers and a decrease in contractility and mobility of these structures. Remarkably, cells expressing the DN PAK still showed extensive ruffling and lamellipodia, though these structures no longer appeared in a polarized manner. Expression of constitutively activated PAK variants also inhibited cell migration. Active PAK also increased stress fibers and focal adhesions, however, these structures retained some degree of dynamic behavior, consistent with increased cell contractility and phosphorylation of MLC.

Cellular responses to the overexpressed PAK constructs show striking cell-type specificity. In HeLa, CHO, and 3T3 cells, active PAK mutants disrupt stress fibers and stimulate lamellipodia formation (Manser et al., 1997, 1998; Sells et al., 1997). Active PAK mutants can also stimulate migration in 3T3 cells (Sells et al., 1999). These effects may be mediated via phosphorylation of MLC kinase by PAK (Sanders et al., 1999) and/or an interaction with PIX (Manser et al., 1998; Daniels et al., 1999). However, a distinct set of effects on cytoskeletal organization occurred in ECs. As we obtained the expected effects on 3T3 cells, it appears that these apparent discrepancies are most likely due to the distinct cell types used in these studies. Most likely, substrates and/or adapter proteins that modulate PAK function differ between cell types.

The effects of PAK constructs on phosphorylation of MLC in HMEC-1 cells were similar to those of Sells et al. (1999) in 3T3 cells, but differ from those of Sanders et al. (1999) in HeLa and BHK cells. The increase in MLC phosphorylation in cells with active PAK is consistent with the measurements of contractility (Fig 10). By contrast, DN PAK caused, if anything, a slight (though statistically insignificant) increase in MLC phosphorylation, but a large decrease in contractility. It also triggered a large increase in actin stress fibers. Thus, DN PAK must induce or stabilize stress fibers by a pathway unrelated to MLC phosphorylation. This idea suggests that PAK may have additional substrates that regulate formation or stability of stress fibers and focal adhesions.

That DN PAK increased stress fibers and focal adhesions, but decreased MLC phosphorylation and contractility may seem to be at odds with the dogma that myosin-generated tension is required for stress fiber formation (Chrzanowska-Wodnicka and Burridge, 1996; Leung et al., 1996; Ishizaki et al., 1997). There is, however, some precedent for this distinction, since caldesmon decreases actomyosin-dependent tension, yet stabilizes actin stress fibers (Pfitzer et al., 1993; Warren et al., 1993). Conversely, active PAK would be predicted to induce a global, nonpolarized increase in contractility. Such an increase could give rise to enhanced actin stress fibers, due to the increase in tension (Byers et al., 1984; Chrzanowska-Wodnicka and Burridge, 1996). However, the nonpolarized nature of the contraction could prevent efficient cell movement.

Past studies of migration suggested a link between extension at the leading edge and retraction at the trailing edge (Kolega, 1986; Palacek et al., 1998; Sheetz et al., 1998). Productive movement requires that cells extend processes, make new adhesions at the front, and then detach old adhesions at the rear of the cell. Detachment may occur by ripping of the cell membrane to leave integrins and actin bound to the substratum, indicating that substantial tension is required (Palacek et al., 1998). In constitu-
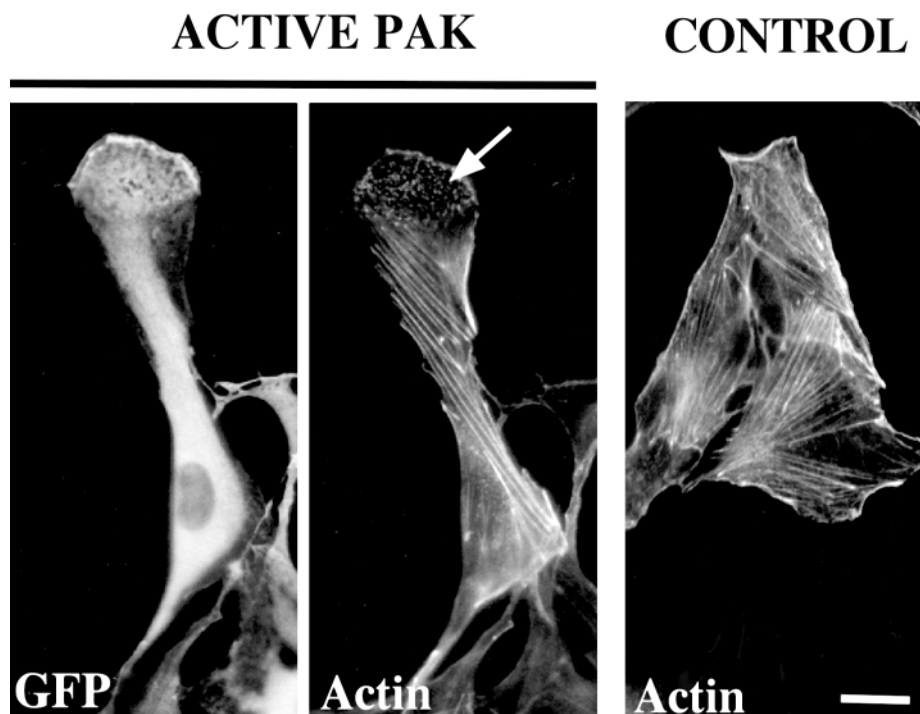

Figure 12. Active PAK in NIH 3T3 fibroblasts. Fluorescence micrographs of NIH 3T3 fibroblasts stained for actin after injection with GFP and H83L,H86L PAK (left) or uninjected control cells (right). Cells injected with active PAK show increased lamellipodial formation and disruption of stress fibers within the lamellipodium (arrow). Bar, 15 μm.
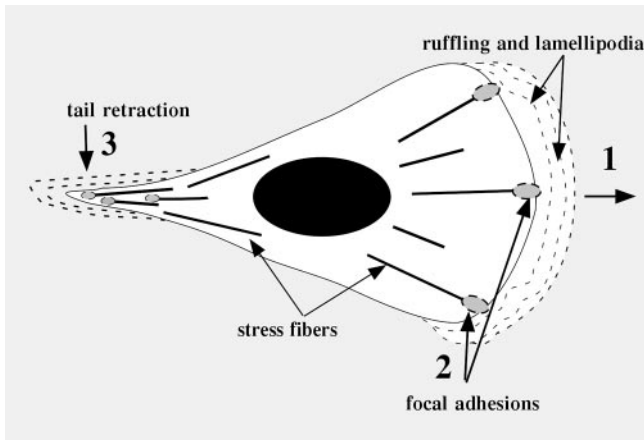
Figure 13. A model for PAK in migration. A cartoon illustrates how PAK may coordinate EC migration. A cell migrates in the direction of predominant ruffling and lamellipodial extension (1). Extension of lamellipodia is followed by formation of new focal adhesions (2), leading to retraction of the tail of the cell at the trailing edge (3). We propose that PAK mediates the induction of tail retraction after formation of new adhesions. The black solid oval represents the nucleus, the bold lines represent stress fibers, and small outlined ovals represent focal adhesions (as indicated by the arrows). Dynamic events are shown as dotted lines.

tively adhesive cells, such as fibroblasts and ECs, these events appear to occur in cycles. Extension of a lamellipodium at the leading edge (Fig. 13, 1) leads to formation of new adhesions at the front of the cell (Fig. 13, 2). New adhesions then initiate signals that stimulate contraction and detachment at the rear of the cell (Fig. 13, 3). Detachment then allows new protrusive activity at the leading edge. In ECs injected with α-actinin, we also observed cycles where lamellipodial extension and focal adhesion formation preceded the initiation of tail detachment, which was followed by new lamellipodia formation (our unpublished observations).

These considerations suggest a model for the role of PAK in EC migration. It may be highly relevant that PAK is activated by formation of new adhesions and is inactivated by detachment (Fig. 2; Price et al., 1998). Thus, formation of new adhesions (Fig. 13, 2) should trigger an increase in PAK activity. We hypothesize that this stimulation leads to contraction and detachment of the cell's tail. Thus, inhibition of detachment is the most likely target of DN PAK. This idea fits the observation that in ECs, DN PAK decreases, whereas active PAK appears to increase, contractility. There are several aspects of this model that remain to be tested. In particular, localization of PAK activity (as opposed to total protein) and its role in localized contraction or focal adhesion stability need to be investigated.

In conclusion, our results indicate that PAK function is required for migration of microvascular ECs. These effects are distinct from those observed in fibroblasts and other cell lines. The available data are consistent with a model in which PAK is not required for formation of lamellipodia or filopodia by Rac or Cdc42, but instead plays a role in coordinating leading edge adhesion formation and trailing

edge detachment to produce polarized cell movement. The data also raise the possibility that PAK may be a suitable target for pharmacological inhibitors of angiogenesis for development of therapies against cancer or other diseases involving pathological angiogenesis.

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