Activation of Rac and Cdc42 by Integrins Mediates Cell Spreading

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Adhesion to ECM is required for many cell functions including cytoskeletal organization, migration, and proliferation. We observed that when cells first adhere to extracellular matrix, they spread rapidly by extending filopodia-like projections and lamellipodia. These structures are similar to the Rac- and Cdc42-dependent structures observed in growth factor-stimulated cells. We therefore investigated the involvement of Rac and Cdc42 in adhesion and spreading on the ECM protein fibronectin. We found that integrin-dependent adhesion led to the rapid activation of p21-activated kinase, a downstream effector of Cdc42 and Rac, suggesting that integrins activate at least one of these GTPases. Dominant negative mutants of Rac and Cdc42 inhibit cell spreading in such a way as to suggest that integrins activate Cdc42, which leads to the subsequent activation of Rac; both GTPases then contribute to cell spreading. These results demonstrate that initial integrin-dependent activation of Rac and Cdc42 mediates cell spreading.

INTRODUCTION

Most cell types respond to surfaces coated with ECM proteins by adhering and then spreading out to acquire a flattened morphology. This process of cell adhesion and spreading is mediated by integrins and involves complex dynamic rearrangements of the actin cytoskeleton. These dynamics appear to be coordinated in space and time by intracellular signaling pathways involving tyrosine kinases, protein kinase C, arachidonic acid metabolism, and, in some cases, intracellular calcium (Chun and Jacobson, 1992, 1993; Pelletier *et al.*, 1992; Auer *et al.*, 1993; Vuori and Ruoslahti, 1993; Romer *et al.*, 1994). How specific signaling pathways regulate the cytoskeleton is, however, poorly understood.

Cells spread by putting out extensions that contact the surface, form adhesions, and then exert tension to induce outward movement. This process is reminiscent of the extensions and adhesions induced by the small GTP-binding proteins Rac and Cdc42. These proteins are closely related members of the Ras superfamily of GTPases, which, like other Ras family members, act as guanine nucleotide-regulated switches. Cdc42 mediates formation of long, thin, actin-dependent extensions called filopodia, whereas Rac mediates formation of curtain-like extensions called lamellipodia and ruffles (Ridley *et al.*, 1992; Nobes and Hall, 1995). Both can induce formation of small substrate adhesions called focal complexes.

Rac and Cdc42 interact with a number of effector proteins. The best characterized effectors are the p21-activated kinases (PAKs). Both Rac and Cdc42 in the GTP-bound state interact specifically with PAKs and strongly stimulate PAK kinase activity (Manser *et al.*, 1994; Knaus *et al.*, 1995; Martin *et al.*, 1995). Mutants of Rac and Cdc42 that do not bind and activate PAK1 can still induce lamellipodia and filopodia, respectively (Joneson *et al.*, 1996; Lamarche *et al.*, 1996), however, activated PAK1 mutants themselves induce lamellipodia and cytoskeletal rearrangements (Sells *et al.*, 1997). Thus, the role of PAK1 in mediating effects of small GTPases on the cytoskeleton is presently unclear. In addition to effectors that are common to Rac and Cdc42, there are molecules such as WASP, POR-1, and p120ACK that interact with one or the other specifically, but the functions of these proteins are even less well defined (for review, see Tapon and Hall, 1997).

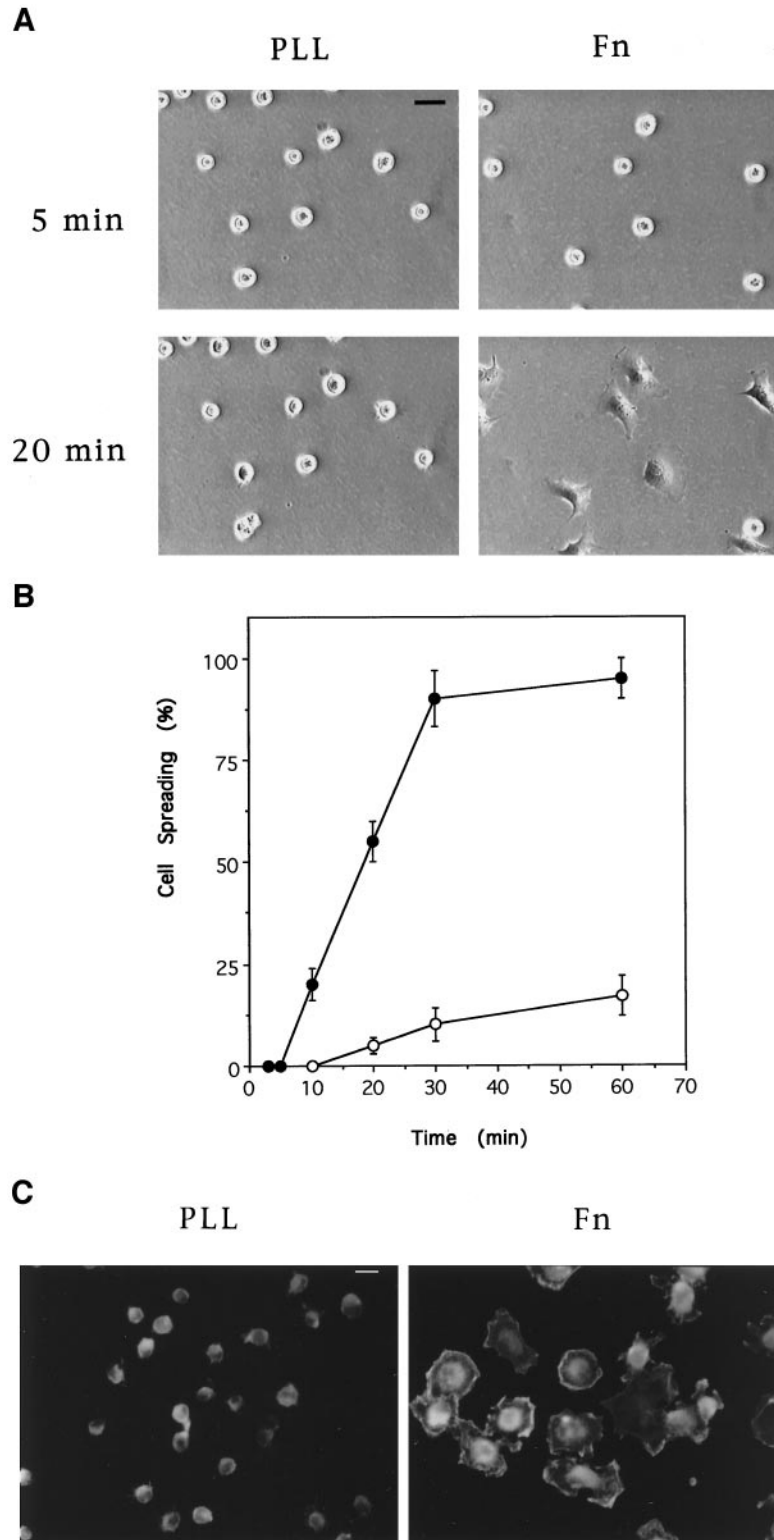‡ Corresponding author. E-mail address: schwartz@scripps.edu.

A

PLL   Fn

5 min

20 min

B

C

PLL   Fn

Figure 1. Cell spreading on Fn and poly-L-lysine. Quiescent cells in suspension were plated on coverslips coated with Fn or poly-L-lysine (PLL). Cells were fixed at the indicated times after plating and visualized by phase contrast microscopy (A) and scored for the percentage of spread cells (B) (●, Fn; ○, poly-L-lysine). (C) Twenty minutes after plating, cells were fixed and permeabilized, and filamentous actin was labeled with rhodamine–phalloidin; cells were then visualized by fluorescence microscopy. Bar, 20 μm.
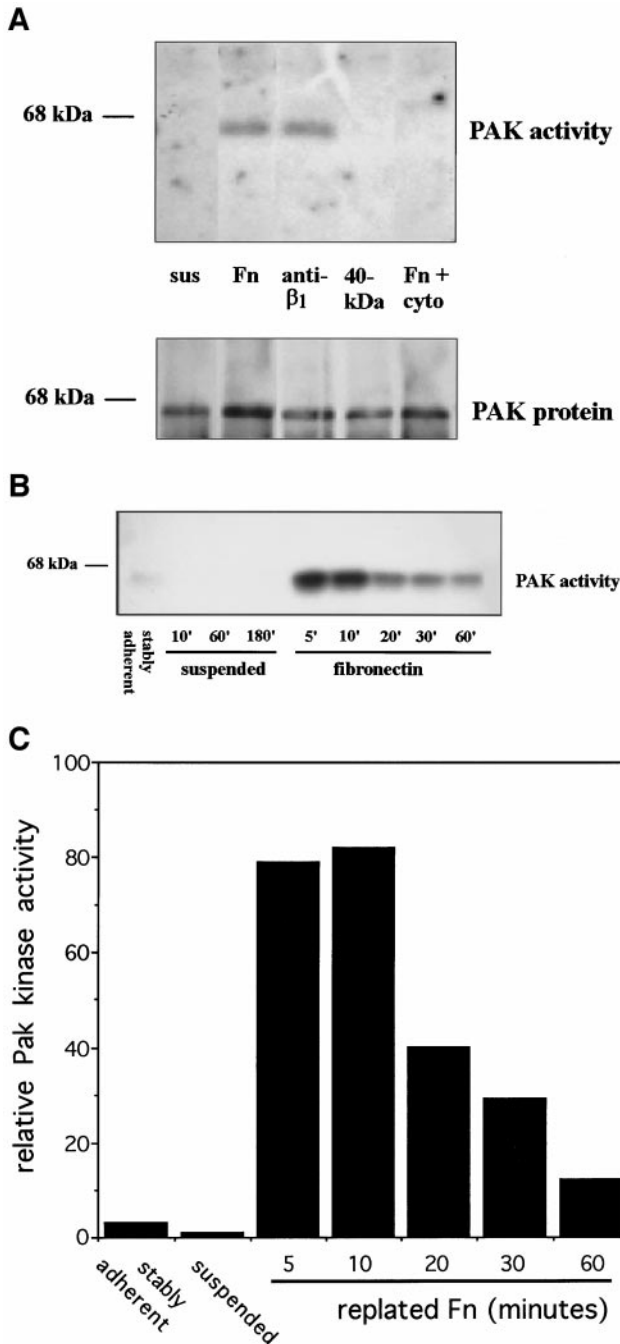
A

68 kDa —   PAK activity

sus   Fn   anti-   40-   Fn +
              β1   kDa   cyto

68 kDa —   PAK protein

B

68 kDa —   PAK activity

stably
adherent 10' 60' 180'   5' 10' 20' 30' 60'
          suspended   fibronectin

C

The similarity between cell spreading and Rac-induced lamellipodia formation prompted us to investigate the role of these small GTPases in cell spreading. Our results indicate that integrins activate these proteins and that both Rac and Cdc42 contribute to cell spreading.

MATERIALS AND METHODS

Proteins and Plasmids

Fibronectin (Fn) was purified from human plasma by affinity chromatography on gelatin-Sepharose (Miekka *et al.*, 1982). Fn 40-kDa α-chymotryptic fragment was purchased from Life Technologies (Gaithersburg, MD). The anti-β1-integrin antibody HMβ1-1 was purchased from PharMingen (San Diego, CA). Myelin basic protein (MBP) was purified from bovine spinal cord as described (Deibler *et al.*, 1972). NP-40 and leupeptin were purchased from ICN Biomedicals (Aurora, OH). Other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Rac mutants were in a pcDNA3 vector; Cdc42 mutants were in pCMV5 (Zhang *et al.*, 1995); and PAK mutants were in pCMV6 (Sells *et al.*, 1997). The GFP vector was from Clontech (Palo Alto, Ca).

Microscopy

Cells were made quiescent by maintaining them in DMEM containing 0.5% serum for 24 h. Quiescent cells were trypsinized, washed, resuspended in serum-free DMEM, and plated on Fn- or poly-L-lysine-coated coverslips. Microscope images were collected continuously on a Panasonic video recorder. For quantification of spreading, cells were fixed in 3.7% formaldehyde at each time point, and the proportion of spread cells was determined under light microscopy. To visualize membrane ruffles, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and actin filaments were stained with rhodamine–phalloidin (0.1 μg/ml) for 30 min. Coverslips were mounted in Fluoromount G and viewed by fluorescence microscopy.

Microinjection and Respreading

NIH 3T3 cells on coverslips were injected with cDNAs coding for various inhibitors of Cdc42 and Rac as described in the text. In some cases, DNA coding for GFP was included at 0.02 μg/ml to allow identification of injected cells. cDNAs were injected into the nucleus according to the method of Meredith *et al.* (1995). Dishes were returned to the incubator for 4 h to allow protein expression. Cells were incubated in trypsin-EDTA sufficiently to induce rounding without detachment, and then the trypsin was carefully aspirated, and fresh medium with 10% serum was added to stop the trypsin. Cells were returned to the incubator for 1 or 4 h as indicated and then fixed with 2% formaldehyde. They were stained for actin filaments with rhodamine–phalloidin (Molecular Probes, Eugene, OR) used at 0.1 μg/ml. Injected cells were identified either by GFP fluorescence or by staining for the myc-tagged dominant negative proteins with a monoclonal anti-myc antibody (9E10) and fluorescein-conjugated sheep anti-mouse secondary antibody. Both methods gave identical results.

Kinase Assays

To assay PAK kinase activity, 70% confluent NIH 3T3 fibroblasts were serum starved in DMEM with 0.5% bovine calf serum for 24 h. Where indicated, cells were trypsinized and suspended for three hours in serum-free DMEM containing 0.1% BSA (protease free) and 0.25 mg/ml soybean trypsin inhibitor. In some cases, cells were then pelleted by centrifugation, rinsed, and extracted in lysis buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 5 mM EDTA, 3 mM
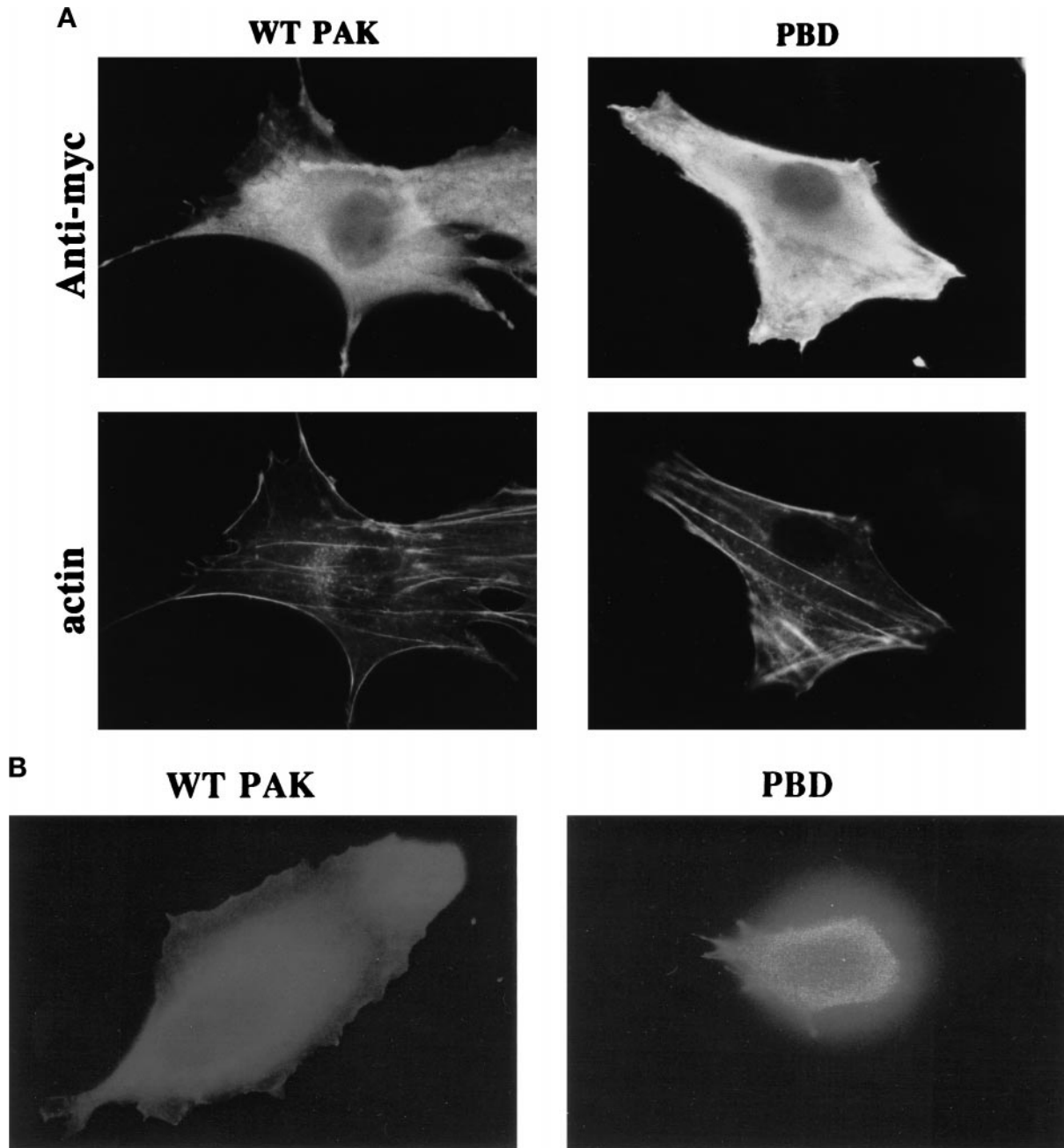
Figure 2. PAK kinase activity is stimulated after integrin-mediated cell adhesion. Cells were trypsinized, placed in suspension, and then plated on tissue culture dishes coated with the indicated ligands. PAK protein was immunoprecipitated, and its kinase activity was assayed. (A) Cells in suspension for 3 h (sus), replated for 20 min on Fn, anti-β1-integrin antibody, 40-kDa Fn fragment, or plated on Fn after a 30-min pretreatment with cytochalasin D (1 μM). The bottom panel shows the amount of PAK protein in immunoprecipitates as determined by Western blotting. (B) Stably adherent cells or cells suspended and replated on Fn for the indicated periods. (C) Densitometric quantification of B showing induction of kinase activity relative to suspended cells.

A

WT PAK   PBD

Anti-myc

actin

B

WT PAK   PBD

Figure 3. The PBD domain of PAK inhibits cell spreading. cDNAs of myc-tagged wild-type PAK (0.5 μg/ml) or the PBD domain of PAK (0.2 μg/ml) were injected into the nuclei of cells. (A) Labeling of expressed myc-tagged proteins with anti-myc antibody and of filamentous actin with rhodamine–phalloidin in stably spread cells after 4 h. (B) Respreading of injected cells for 1 h after mild trypsinization; expression of myc-tagged proteins is demonstrated by anti-myc staining. (C, facing page) Quantification of cell spreading. Values are means ± SD from three experiments in which >25 cells were scored per condition.

EGTA, 20 mM sodium phosphate, 10 mM sodium pyrophosphate, 3 mM β-glycerophosphate, 10 μg/ml leupeptin, 1 mM sodium vanadate, 1 mM PMSF, 1 mM NaF). Alternatively, cells were transferred to dishes that had been coated with 25 μg/ml Fn, anti-β1 immunoglobulin G, or the 40-kDa fragment of Fn and then blocked with 1% heat-denatured BSA. Cells were allowed to adhere for the

indicated period, rinsed two times with cold PBS, and extracted in lysis buffer.

Endogenous PAK was immunoprecipitated from 150–250 μg cell lysate with anti-PAK1 antibodies (polyclonal anti-PAK1 R626; Dharmawardhane *et al.*, 1997) and dissolved in SDS-sample buffer. To estimate the amount of immunoprecipitated PAK, one-fifth of
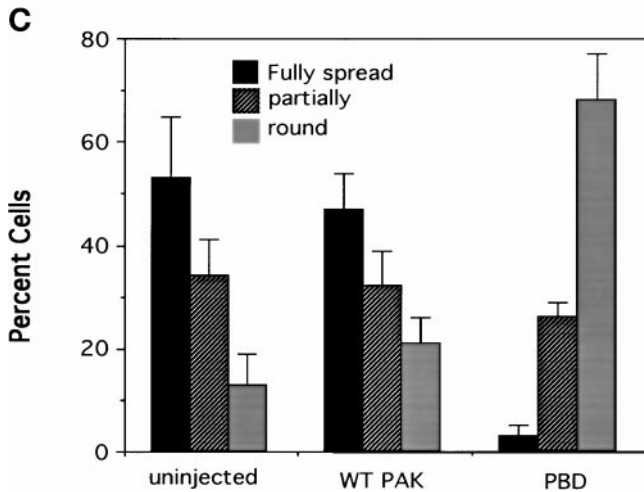
Figure 3 (cont).

each sample was run on 10% SDS-polyacrylamide gels and proteins transferred to nitrocellulose (Hybond C; Amersham, Little Chalfont, United Kingdom). Membranes were probed with the anti-PAK antibody, and protein was detected by enhanced chemiluminescence. PAK kinase activity in the immunoprecipitates was determined using an in-gel kinase assay as described previously (Renshaw *et al.*, 1996). Briefly, immunoprecipitates were run on 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP. Proteins in the gel were then renatured, and their kinase activity toward MBP was initiated by soaking the gels in kinase buffer containing 25 μCi/ml [γ-^{32}P]ATP and 10 μM unlabeled ATP. Gels were then washed extensively and autoradiographed, and films were scanned by densitometry using an Alphaimager 2000 (Alpha Innotech, San Leandro, CA).

RESULTS

Adhesion to Fn Stimulates Spreading and Membrane Ruffling

NIH 3T3 fibroblasts plated on Fn-coated surfaces adhere within ~5 min and then spread over a period of ~1 h (Figure 1, A and B). During this period, cells extend filopodia and lamellipodia and show extensive membrane ruffles (Figure 1C). Similar structures observed in growth factor-stimulated cells are mediated by the small GTPases Cdc42 and Rac, respectively (Ridley et al., 1992; Nobes and Hall, 1995). These results suggest that, in this cell type, Rac and or Cdc42 may be activated during cell spreading.

Integrin Activation of the Rac and Cdc42 Effector PAK

Direct assays of Rac or Cdc42 activation are technically difficult; therefore, to investigate possible integrin dependence of Rac and/or Cdc42 activity, we assayed the serine/threonine kinase PAK, which is a direct downstream target of these GTPases (Manser *et al.*, 1994; Knaus *et al.*, 1995). Cells were incubated for 24 h in 0.5% serum to minimize growth factor activa-

tion and then were detached and incubated in serum-free medium. After 3 h in suspension, cells had minimal PAK kinase activity, but plating on Fn-coated tissue culture dishes strongly stimulated PAK (Figure 2A). Activation was also observed when cells were plated on an antibody to β1-integrins but not on dishes coated with the 40-kDa tryptic fragment of Fn, to which cells adhere via heparin sulfate proteoglycans (Woods *et al.*, 1993). Consistent with the presence of PAK kinase activity, cells placed on Fn or anti-β1 antibodies extended processes and spread, whereas cells plated on the Fn 40-kDa fragment remained completely rounded (our unpublished results). Cell spreading and PAK activation were also observed when cells were plated on vitronectin. Pretreatment with cytochalasin D before plating cells on Fn prevented activation of PAK (Figure 2A), indicating that organization of the actin cytoskeleton is essential for integrin-mediated activation of this pathway. Cytochalasin D treatment also blocked cell spreading. These results demonstrate that integrin-dependent adhesion specifically activates PAK and, by inference, Rac and/or Cdc42.

Examination of the time course of PAK activation showed that kinase activity was nearly maximal at 5 min (the earliest time point measured), peaked at 10 min, and fell to a near-baseline level of activity after ~1 h (Figure 2, B and C). Thus, activation of this pathway is an early response to cell adhesion that precedes cell spreading.

Effect of Inhibitors of Rac and Cdc42 on Cell Spreading

To investigate whether activation of Rac and/or Cdc42 is required for cell spreading, cells were microinjected with cDNAs encoding epitope-tagged dominant negative mutant proteins, which inhibit these GTPases. We first examined the Rac and Cdc42 binding domain of PAK (p21 binding domain [PBD]) that binds and sequesters both Rac and Cdc42 and prevents their interaction with downstream effectors (Sells *et al.*, 1997). Expression of neither PBD nor wild-type PAK (which was used as a control) had any detectable effect on cell morphology or actin distribution in stably adherent cells after 4 h (Figure 3A). Expression of the proteins was confirmed by staining with an antibody against the epitope tag. To enable examination of spreading in microinjected cells, cells were induced to round up (but not detach) by brief trypsinization. The trypsin was stopped, and cells were allowed to respread for 1 h. Expression of wild-type PAK had no effect, but expression of PBD strongly inhibited respreading of cells in this assay (Figure 3, B and C). These results indicate that Rac and/or Cdc42 are required for cell spreading.
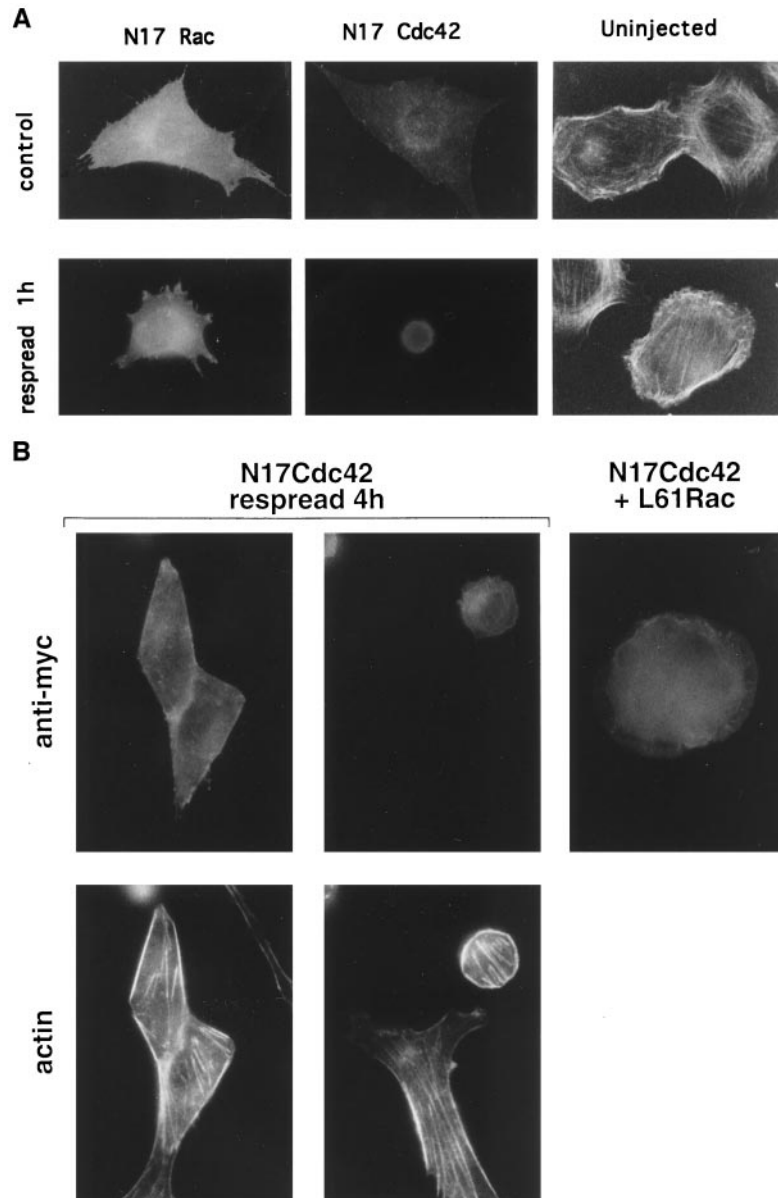
Figure 4. Effect of N17Rac and N17Cdc42 on cell spreading. N17Rac and N17Cdc42 cDNAs (0.2 μg/ml) were injected into the nuclei of cells. (A) Effect of expressed proteins on stably adherent cells (control) and cells allowed to respread for 1 h after mild trypsinization; anti-myc staining of injected cells and rhodamine–phalloidin staining of uninjected cells is shown. (B) Cells injected with N17Cdc42 and allowed to respread for 4 h or cells injected with N17Cdc42 plus constitutively active Rac (L61Rac) (0.5 μg/ml) and allowed to respread for 1 h. (C, facing page) Quantification of cell spreading. Values are means ± SD from four or five experiments in which >25 cells were scored per condition. The difference between N17 Rac and control cells is statistically significant ($p < 0.005$).

To determine to what extent Rac or Cdc42 or both mediate spreading, cells were injected with cDNAs encoding the dominant negative mutants N17Rac and N17Cdc42. Expression of these proteins in stably adherent cells did not cause gross changes in morphology or cytoskeletal structure, although N17Rac-expressing cells showed a loss of lamellipodia and an increase in filamentous projections, which were most likely either retraction fibers or filopodia (Figure 4A). Expression of N17Rac caused a partial inhibition of respreading of rounded cells (Figure 4, A and C). Notably, cells appeared to spread by means of narrow extensions instead of the usual broad lamellipodia. It should be noted that expression of fourfold lower levels of N17 Rac completely inhibited PDGF-induced ruffling, suggesting that the concentration of N17 Rac
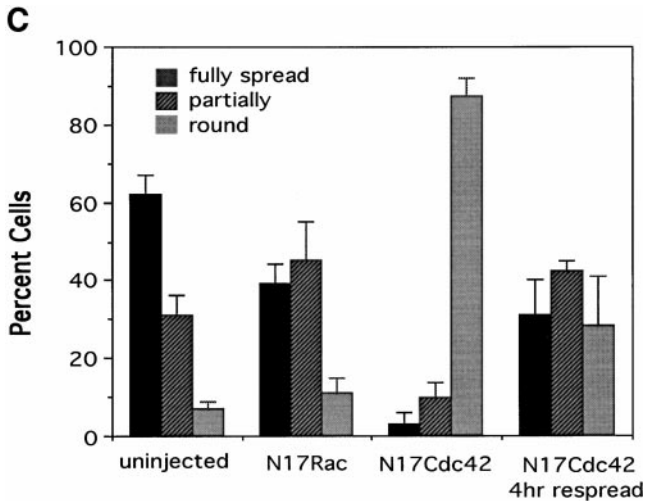
Rac and Cdc42 Mediate Spreading
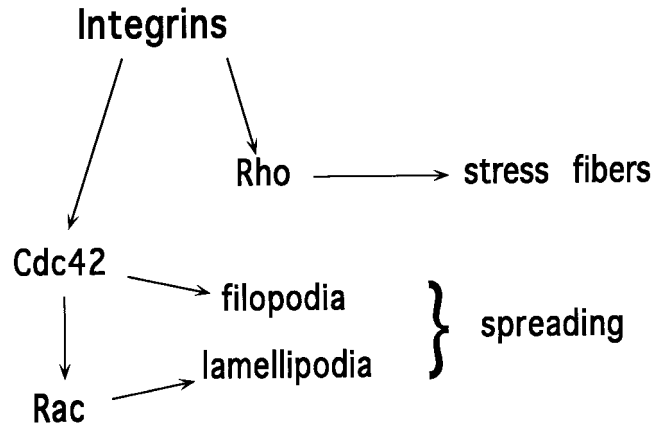
C

Figure 4 (cont).

Figure 5. Schematic diagram showing integrin-dependent activation of Rho family GTPases. Engagement of integrins with ECM leads to the activation of Cdc42, which subsequently leads to the activation of Rac. Together, these GTPases mediate cell spreading. Activation of Rho can occur independently of activation of Cdc42 and leads to the formation of stress fibers.

DNA used in these experiments should be effective. By contrast, N17Cdc42 profoundly inhibited respreading; after 1 h, almost all injected cells remained completely round.

The inhibition by N17Cdc42 was so dramatic that additional controls were performed. To determine whether the inhibition of respreading by N17Cdc42 was reversible, cells were left to recover for longer periods. When cells were allowed to recover for 4 h, the majority of cells showed some degree of respreading (Figure 4, B and C). Interestingly, even those cells that failed to respread developed actin stress fibers (Figure 4B). Stress fibers have been shown to be a consequence of Rho activation, suggesting that Rho activation is independent of Cdc42. These results indicate that N17Cdc42-expressing cells remained viable and capable of assembling actin-based structures that are independent of Cdc42.

It has been demonstrated in some systems that Cdc42 can lead to activation of Rac (Nobes and Hall, 1995). Thus, the nearly complete inhibition of spreading by N17Cdc42 could be explained if initial activation of Cdc42 by integrins induced both Cdc42- and Rac-dependent events. To determine whether the inhibition of spreading by N17Cdc42-was due to the loss of Cdc42 activity alone or the additional loss of signaling to Rac, N17Cdc42 was expressed along with a constitutively activated mutant of Rac, L61Rac. Coexpression of activated Rac partially restored the ability of cells to respread after 1 h (Figure 4B). Quantification of these results showed that L61Rac increased the total number of spread cells (full plus partial) threefold from 15.2 ± 5.9 to $48.7 \pm 18.1\%$ (p < 0.005; n = 5). This result further demonstrates that N17Cdc42 is not toxic and suggests that Rac lies downstream of Cdc42 in this system.

DISCUSSION

Plating cells on Fn or antibodies to integrins leads to cell spreading and membrane ruffling. This result suggested that the small GTPase Rac was activated. We therefore investigated whether integrins activated Rac and whether this activation was involved in cell spreading.

The serine/threonine kinase PAK is activated directly by Rac and Cdc42 (Manser *et al.*, 1994; Knaus *et al.*, 1995; Martin *et al.*, 1995). We therefore assayed PAK kinase activity as an indicator of GTPase activation. PAK kinase activity was rapidly induced upon plating cells on Fn or anti-β1-integrin immunoglobulin G, suggesting that Rac and/or Cdc42 was indeed activated. PAK was not activated when cells adhered to a control protein that does not bind integrins, demonstrating a specific requirement for integrins. The time course of activation was rapid, with almost maximal kinase activity at 5 min. At this early time point, cell spreading is either minimal or absent, suggesting that Rac and/or Cdc42 activation precedes cell spreading.

To test whether Rac and Cdc42 were required for integrin-mediated cell spreading, we expressed the PBD domain of PAK, which inhibits these GTPases (Sells *et al.*, 1997). We found that the PBD domain profoundly inhibited cell spreading, demonstrating that Rac and/or Cdc42 GTPases are essential. To determine which of the two GTPases were required, we expressed dominant negative inhibitory forms of the proteins. We observed that dominant negative Rac retarded cell spreading, demonstrating a role for Rac. In the presence of N17Rac, cells spread by extending thin processes instead of lamellipodia, suggesting that

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spreading occurred via Cdc42. Dominant negative Cdc42 profoundly inhibited spreading, with nearly all cells appearing completely round at 1 h, demonstrating a pivotal role for this protein. The deficiency in spreading in cells expressing N17Cdc42 could be overcome with time. This may have occurred via a Cdc42-independent mechanism, perhaps by the independent activation of Rac, or may be a consequence of incomplete inhibition of Cdc42. Interestingly, even in those cells that did not significantly spread, stress fibers still formed, indicating that Rho function was not dependent on Cdc42 or Rac.

To determine whether integrins activated Cdc42 and Rac independently or whether activation of these proteins was linear, we coexpressed N17Cdc42 with activated Rac. Activated Rac partially reversed the inhibition caused by N17Cdc42. As might be expected, the cells that spread did so by extending broad, symmetrical lamellipodia indicative of Rac activation (Ridley *et al.*, 1992). The incomplete recovery of spreading could be because Rac activation was not properly coordinated in space and time or because Cdc42 contributes something distinct from Rac that enhances spreading. Despite these uncertainties, the data suggest that integrin engagement leads to the activation of Cdc42, which results in the subsequent activation of Rac, and that both GTPases contribute to cell spreading.

Our results show that adhesion to ECM stimulates the activation of PAK. However, published data as to the role of PAK in Rac- and Cdc42-dependent control of cell architecture has been contradictory. PAK has been shown to colocalize with Rac and Cdc42 at focal adhesions and membrane ruffles (Harden *et al.*, 1996; Dharmawardhane *et al.*, 1997) and to induce filopodia and lamellipodia when injected into Swiss 3T3 cells (Sells *et al.*, 1997). Other studies, however, showed that mutations in Rac and Cdc42 that abolish binding to PAK in vitro did not block the ability to induce the formation of lamellipodia and filopodia (Joneson *et al.*, 1996; Lamarche *et al.*, 1996). To determine whether PAK is a functionally important effector in this system, the activated mutant PAK 83/86 (Sells et al., 1997) was coexpressed with N17 Cdc42. No recovery of cell spreading after trypsinization was observed, indicating that PAK is not sufficient for spreading in the absence of other effectors. These experiments do not, however, exclude the possibility that PAK could influence events triggered by other effectors. Thus, the function of PAK in GTPase-regulated cytoskeletal regulation is still unclear.

These results suggest a model for cell spreading whereby initial contact with ECM proteins activates Cdc42 and induces the extension of filopodial processes. Activation of Cdc42 leads to the subsequent activation of Rac and the formation of lamellipodia, which extend between the filopodia. Rho is activated independently to induce stress fibers and generate tension. This model is shown in Figure 5. It is notable that this model proposes a molecular mechanism for cell spreading that closely resembles cell migration, with the exception that instead of the unidirectional extension of filopodia and lamellipodia toward a chemotactic stimulus, processes extend isotropically to increase cell area.

We have shown here that integrins induce Rac- and Cdc42-mediated cell spreading in the absence of exogenous growth factors. By contrast, adherent serum-starved Swiss 3T3 cells do not possess stress fibers or membrane ruffles and require growth factors or addition of activated Rho and Rac to induce these structures (Ridley and Hall, 1992; Ridley *et al.*, 1992). These differences may be due to basic differences in the signaling pathways between the two cell types. However, it has been shown that if the level of integrin occupancy in starved Swiss 3T3 cells is increased by replating cells on Fn or by adding the Fn cell-binding peptide GRGDS, cells do spread and form Rho-dependent stress fibers (Allen *et al.*, 1997). These results suggest that it is primarily the extent of integrin occupancy required to induce activation of GTPases that differs between the two cell types.

Adhesion is a basic requirement for proliferation of most cell types. It has been proposed that constitutive activation of signaling pathways that are normally activated by integrins may overcome the adhesion requirement for cell growth (Schwartz, 1993; Schwartz *et al.*, 1996). Recent results showing that activated Cdc42 confers anchorage-independent growth but that cells remain serum dependent (Qiu *et al.*, 1997) therefore support our conclusion that integrins activate Cdc42. These results further suggest that integrin-mediated adhesion may therefore contribute to cell proliferation via the activation of Cdc42.

Evidence has also been presented that Rho is activated by integrins and that enhanced activation of Rho by overexpression of Rho nucleotide exchange factors can also confer anchorage independence (Chong *et al.*, 1994; Schwartz *et al.*, 1996). How integrins activate Cdc42 and Rho is not known, but the most obvious hypothesis is that they may activate nucleotide exchange factors for these GTPases. Although not yet identified in NIH 3T3 cells, exchange factors have been identified in other cell types that act on Cdc42 and Rho (Horii *et al.*, 1994; Olson *et al.*, 1996). Regulation of these factors by integrins may therefore be a useful direction for future investigations.

ACKNOWLEDGMENTS

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