

Integrin-mediated Signals Regulated by Members of the Rho Family of GTPases

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Abstract. The organization of the actin cytoskeleton can be regulated by soluble factors that trigger signal transduction events involving the Rho family of GTPases. Since adhesive interactions are also capable of organizing the actin-based cytoskeleton, we examined the role of Cdc42-, Rac-, and Rho-dependent signaling pathways in regulating the cytoskeleton during integrin-mediated adhesion and cell spreading using dominant-inhibitory mutants of these GTPases. When Rat1 cells initially adhere to the extracellular matrix protein fibronectin, punctate focal complexes form at the cell periphery. Concomitant with focal complex formation, we observed some phosphorylation of the focal adhesion kinase (FAK) and Src, which occurred independently of Rho family GTPases. However, subsequent phosphorylation of FAK and paxillin occurs in a Rho-dependent manner. Moreover, we found Rho dependence of the assembly of large focal adhesions from which actin stress fibers radiate. Initial adhesion to fibronectin also stimulates membrane ruffling; we show that this ruffling is independent of Rho but is depen-

dent on both Cdc42 and Rac. Furthermore, we observed that Cdc42 controls the integrin-dependent activation of extracellular signal-regulated kinase 2 and of Akt, a kinase whose activity has been demonstrated to be dependent on phosphatidylinositol (PI) 3-kinase. Since Rac-dependent membrane ruffling can be stimulated by PI 3-kinase, it appears that Cdc42, PI 3-kinase, and Rac lie on a distinct pathway that regulates adhesion-induced membrane ruffling. In contrast to the differential regulation of integrin-mediated signaling by Cdc42, Rac, and Rho, we observed that all three GTPases regulate cell spreading, an event that may indirectly control cellular architecture. Therefore, several separable signaling pathways regulated by different members of the Rho family of GTPases converge to control adhesion-dependent changes in the organization of the cytoskeleton, changes that regulate cell morphology and behavior.

Key words: integrins • adhesion • cytoskeleton • Rho • Cdc42

ADHESIVE interactions play an important role in regulating numerous cellular functions during the development and maintenance of an organism, and alterations in these interactions can lead to numerous disorders. In tissue culture, cells adhere to the underlying extracellular matrix (ECM)¹ via integrins, a family of cell surface receptors, at discrete sites termed “focal adhesions” (Burridge and Chrzanowska-Wodnicka, 1996; Craig and Johnson, 1996; Jockusch and Rudiger, 1996). Integrins

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; Erk, extracellular signal-regulated kinase; F-actin, filamentous actin; FAK, focal adhesion kinase; HA, hemagglutinin; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; PI, phosphatidylinositol; SH2, Src homology 2.

then link the ECM to large bundles of intracellular microfilaments that form prominent “stress fibers” in many cell types. When cells are plated on an ECM-coated surface, complex series of events are set in motion that contribute to the establishment of focal adhesions and actin stress fibers. Adhesion also affects additional actin-based structures such as microspikes, membranous ruffles, and morphological changes involved in cell spreading and motility (Craig and Johnson, 1996; Zigmond, 1996).

Integrin engagement and subsequent clustering of these receptors in focal adhesions leads to the generation of intracellular macromolecular complexes (Yamada and Miyamoto, 1995). Numerous proteins present at the cytoplasmic face of focal adhesions are considered to be structural components of focal adhesions, including cytoskeletal proteins such as vinculin and talin (Jockusch and Rudiger, 1996). In addition, numerous “signaling” proteins with en-

zymatic activity (e.g., kinases and GTPases) are also components of focal adhesions. By clustering these structural and signaling components together, integrins transduce the signals required to mediate certain aspects of cell physiology and morphology (Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Geiger, 1997). These signals may include tyrosine phosphorylation of proteins such as paxillin and p130CAS (BurrIDGE et al., 1992; Petch et al., 1995), activation of protein tyrosine kinases such as the focal adhesion kinase (FAK) and Src (Schaller and Parsons, 1994; Kaplan et al., 1995), and activation of serine/threonine kinases such as the extracellular signal-regulated kinases (Erks) or Akt (Chen et al., 1994; Schlaepfer et al., 1994; King et al., 1997).

The Rho family of GTPases appears poised to contribute to these integrin-mediated signals; in particular, signals that control cytoskeletal organization involved in changes in cell morphology. Rho family members such as Cdc42, Rac1, and RhoA are part of the Ras superfamily of proteins that cycle between an active, GTP-bound state and an inactive, GDP-bound state. Activated RhoA is capable of stimulating microfilament bundling in serum-starved cells that are already adherent (Ridley and Hall, 1992), similar to the response of cells to plating on an ECM-coated surface. Rho is also essential for the formation of focal complexes (Hotchin and Hall, 1995). The Rho-family member Rac controls growth factor-stimulated membrane ruffling and formation of lamellipodia (Ridley et al., 1992). Finally, Cdc42 activation triggers the extension of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). Studies such as these have defined how soluble extracellular factors induce the assembly of focal adhesions and stress fibers in serum-starved adherent Swiss 3T3 fibroblasts through activation of the Rho family of GTPases (Ridley, 1996; Tapon and Hall, 1997).

Considerably less is known about how the ECM acts as an insoluble extracellular agent capable of inducing the assembly of these same structures when cells are plated on a matrix. For instance, is the Rho family of GTPases involved in the generation of these morphological structures? Initial studies suggested that in Swiss 3T3 cells integrin engagement of the matrix alone was not sufficient to induce the assembly of vinculin-containing focal adhesions; Rho must also be activated by a soluble factor (Hotchin and Hall, 1995). More recently it was shown that serum-starved Swiss 3T3 cells can form Rho-dependent focal adhesions in the absence of added soluble factors if they are given sufficient time (Barry et al., 1997). Therefore, Rho activity is essential for focal adhesion formation when cells are plated on a matrix, suggesting that Rho is involved in signaling from integrin receptors. While these studies have begun to define the mechanisms used by cells to generate morphological structures, a role for other Rho family members has yet to be established. Furthermore, the integrin-mediated signaling pathways regulated by the Rho family have yet to be defined in detail. Therefore, our goal in this study was to define the morphological changes and integrin-mediated signaling pathways that are regulated by the Rho family GTPases, Cdc42, Rac, and Rho, in response to plating cells on the ECM protein fibronectin, an insoluble stimulus, and in so doing, correlate changes in morphology with changes in signaling.

Materials and Methods

Reagents

Antibodies to paxillin (No. P13520), phosphotyrosine (PY20), and Erk (No. E17120) were purchased from Transduction Laboratories (Lexington, KY). The antibody to the c-Myc tag (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-hemagglutinin (HA) antibody (12CA5) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). The polyclonal antiserum to the COOH-terminal of FAK was a gift from J.-L. Guan (Cornell University, Ithaca, NY). The antibody to Src (phosphotyrosine 416) was a gift from A. Laudano (University of New Hampshire, Durham, NH). The polyclonal antiserum to Akt was provided by P. Tschlis (Fox Chase Cancer Center, Philadelphia, PA). The anti-integrin $\beta 1$ subunit antibody (130) was previously characterized (Marcantonio and Hynes, 1988).

Cell Culture

Rat1 lines expressing N17Rac1 or N19RhoA driven by a tetracycline-repressible promoter were established as described (Qiu et al., 1995a,b). To enhance expression of N17Rac1 or N19RhoA, tetracycline was withdrawn 2 d before the cells were used. Rat1 lines expressing N17Cdc42 were derived as described (Qiu et al., 1997). All cells were maintained at 37°C and 5% CO₂ in DME, supplemented with 10% FBS, 2 mM glutamine, penicillin at 400 units/ml, streptomycin at 150 μ g/ml, and G418 at 400 μ g/ml; puromycin at 2 μ g/ml and tetracycline at 2 μ g/ml were then added to the N17Rac1- and N19RhoA-expressing Rat1 lines. Results observed in the N17Cdc42- and N17Rac1-expressing cell lines were confirmed in two clones, and results from the N19RhoA-expressing line were confirmed by treating Rat1 cells with the C3 transferase. C3 transferase (a gift from M. Schwartz, Scripps Research Institute, La Jolla, CA) was introduced into Rat1 cells with Lipofectamine. C3 transferase (25 μ g) and Lipofectamine reagent (25 μ g) were combined in 1 ml DME and incubated for 10 min at room temperature. An additional 4 ml of DME was then added, and the solution was added to serum-starved cells for 4 h at 37°C. The cells were washed in PBS, trypsinized, and then prepared as described below. C3 transferase treatment caused the cells to retract lamellipodia and roundup, but the cells were viable and able to reattach and spread (see Cell Preparation for Adhesion below).

Transient Transfection Analysis

Calcium phosphate transient transfection of Rat1 cells was performed essentially as described (Sambrook et al., 1989). In brief, 1–11 μ g of plasmid DNA was coprecipitated with calcium phosphate and introduced to a 10-cm dish of cells for 8 h. The cells were then rinsed and maintained in media for an additional 48 h, followed by serum starvation as described below.

Cell Preparation for Adhesion

The cell lines described above were washed in PBS and then starved in serum-free DME for 24 h. Starved cells were trypsinized for 2 min, washed with DME containing 1 mg/ml soybean trypsin inhibitor and 0.1% BSA, and then finally resuspended in DME for 30 min at 37°C before plating the cells on polylysine- (0.5 mg/ml) or fibronectin-coated (10 μ g/ml) dishes or coverslips as previously described (Clark and Hynes, 1996).

Immunofluorescence

Adherent cells were rinsed in PBS, fixed in 4% PFA in PBS, and then permeabilized with 0.5% NP-40 in PBS. Filamentous actin (F-actin) was stained using rhodamine-labeled phalloidin and focal adhesions were stained using an antibody to vinculin (Sigma Chemical Co., St. Louis, MO) or to the integrin $\beta 1$ subunit (rabbit antiserum 130) and a fluorescein-conjugated secondary antibody.

Cell Lysis, Immunoprecipitation, and Immunoblotting

Cells were washed twice in PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EGTA, 1 mM PMSF, 500 μ g/ml leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride) for 30 min on ice. The lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C, and protein concentration was then determined

using a Pierce Micro BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Immunoprecipitations were then performed using 50 μ g (for FAK) or 100 μ g (for paxillin) of RIPA lysate, the appropriate antibody, and protein A-Sepharose. Duplicate immunoprecipitates of FAK and paxillin were immunoblotted with their respective antibodies to ensure that equal amounts of these proteins were present. Total cell lysates were prepared by adding one part 4 \times SDS sample buffer (8% SDS, 2% 2-mercaptoethanol, 266 mM Tris, pH 7.2, 40 mM EDTA) to three parts RIPA lysate. The samples were then heated to 100°C for 5 min and run on SDS-polyacrylamide gels. Immunoblotting of the total cell lysates or immunoprecipitates was performed essentially as described (Clark and Brugge, 1993). Briefly, proteins separated by SDS-polyacrylamide gels were transferred electrophoretically to nitrocellulose, which was blocked for 60 min in 5% nonfat milk. The nitrocellulose was incubated for 120 min in primary antibody, washed for 30 min in Western Wash buffer (150 mM NaCl, 50 mM Tris, pH 7.6, 0.1% NP-40), incubated for 90 min in HRP-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin, and then washed for an additional 30 min in Western Wash buffer. The proteins were detected using a chemiluminescence detection kit (ECL; Amersham Corp., Arlington Heights, IL); three exposures of the immunoblot were taken (15, 60, and 240 s) to insure linearity of the signal and the resulting autoradiographic films quantitatively analyzed using an Is-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

Erk In Vitro Kinase Assay

Cells were washed twice in PBS and lysed in Erk lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 40 mM β -glycerophosphate, 40 mM *para*-nitrophenyl phosphate, 0.2 mM Na₃VO₄, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin). The lysates were cleared by centrifugation at 16,000 *g* for 10 min at 4°C, and protein concentration was determined as described above. Immunoprecipitation was performed with 500 μ g of lysate, 10 μ g of 12CA5, and protein A-Sepharose. The immunoprecipitates were then washed and subjected to an *in vitro* kinase reaction (using myelin basic protein as substrate) as described (King et al., 1997). The specific activity of Erk2 was determined by quantitating the ³²P incorporated into myelin basic protein with a PhosphorImager and dividing it by the level of protein in the precipitate.

Akt In Vitro Kinase Assay

Cells were washed twice in PBS and lysed in Akt lysis buffer (20 mM Tris, pH 7.5, 150 mM sodium chloride, 10% glycerol, 1% NP-40, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 500 μ g/ml leupeptin, 2 mM aprotinin, and 1 mM PMSF). Akt was immunoprecipitated from 250 to 500 μ g of lysate with a polyclonal antibody to Akt or the HA tag antibody (12CA5) and subjected to an *in vitro* kinase reaction using histone H2B as an exogenous substrate as described (King et al., 1997).

Adhesion and Spreading Assays

Serum-starved cells (500,000 cells/ml) suspended in 1 ml of DME as described above were plated on fibronectin-coated/BSA-blocked 35-mm wells. To initiate adhesion, the cells were centrifuged for 30 s at 50 *g* and allowed to adhere (10 min) or spread (20 min) at 37°C. Adherent cells were washed twice with PBS and then fixed with 4% PFA in PBS for 15 min at room temperature. The fixed cells were then rinsed twice with water, stained with 0.1% crystal violet in water for 30 min at room temperature, and rinsed twice with water. Adhesion was quantitated by adding 10% acetic acid to the crystal violet-stained well and examining the solution in a spectrophotometer at 600 nm. Background staining was determined by staining cells that adhere to a BSA-coated well in the absence of fibronectin. Spreading was quantitated by photographing the cells that adhere to fibronectin-coated wells after 20 min and counting the cells that had spread.

Determination of F-Actin Content

F-actin content was examined essentially as described (Southwick et al., 1989). In brief, serum-starved cells (800,000 cells/ml) suspended in 1 ml of DME were plated on fibronectin- or polylysine-coated/BSA-blocked 35-mm wells for 15 or 60 min at 37°C. The cells were then washed twice in PBS and fixed with 4% PFA in PBS for 10 min at room temperature. Next, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, rinsed three times in PBS, and then F-actin was

stained with fluorescein-labeled phalloidin for 30 min at 37°C. After washing the cells three times with PBS, the labeled phalloidin was released from the cells by adding 0.5 ml of methanol to each well. The cells were then shaken for 2 h at room temperature in a dark humidified chamber and 0.3 ml of the solution was removed and measured using a spectrophotometer (excitation and emission wave lengths of 485 and 538 nm, respectively).

Results

Changes in Cell Morphology That Accompany Cell Spreading on Fibronectin

When grown in culture, many cells form specialized adhesive structures that link them to the underlying matrix. During the assembly of these structures, there are dramatic changes in cell morphology. To define the changes in cell morphology that accompany cell adhesion and spreading, serum-starved Rat1 fibroblasts were plated onto fibronectin for various times and stained for focal adhesions and F-actin. Fig. 1 shows 15-min and 60-min time points. Rat1 cells adhered rapidly to fibronectin, with >90% of the cells adhering within 10 min. By 15 min, most of the cells had begun to flatten, with large membranous protrusions (“ruffles”) appearing at the cell perimeter (Fig. 1 *a*). In those cells that had initiated cell spreading, punctate concentrations of vinculin were present at the perimeter in structures (Fig. 1 *b*) that resembled “point contacts” (Tawil et al., 1993) or “focal complexes” (Nobes and Hall, 1995). F-actin was also present at the cell perimeter in membranous protrusions that appeared to be membrane ruffles (Fig. 1 *a*). At this time there were very few cytoplasmic actin filaments that could be visualized. However, at 60 min there were many bundles of actin filaments that spanned the central core of the cell (Fig. 1 *c*), and these bundles appeared to be anchored at the cell periphery in vinculin-containing focal adhesions (Fig. 1 *d*).

These structures that formed when cells adhered and spread on an insoluble ECM protein such as fibronectin closely resembled the structures that form when adherent Swiss 3T3 cells are stimulated with PDGF or serum (Ridley et al., 1992; Ridley and Hall, 1992). To test whether Rat1 cells were also susceptible to activation by these soluble factors, serum-starved adherent Rat1 cells were treated with buffer, PDGF, or FBS for 10 min, and then stained for F-actin. As shown in Fig. 2 *a*, some F-actin-containing structures remained in serum-starved Rat1 cells, but serum treatment of these cells resulted in a dramatic increase in actin bundles (Fig. 2 *c*) and PDGF treatment induced the localization of F-actin into membrane ruffles (Fig. 2 *b*). Therefore, both soluble and insoluble factors are capable of stimulating the formation of cytoskeletal structures such as membrane ruffles, focal adhesions, and actin bundles in Rat1 cells.

Cell Spreading Is Cdc42, Rac, and Rho Dependent

Whereas members of the Rho family of small GTPases are known to regulate the assembly of cytoskeletal structures stimulated by soluble factors, relatively little is known about the role of the Rho family in integrin-mediated changes in cell morphology. To examine this, we used Rat1 cells that inducibly express dominant-inhibitory mutants of the Rho family members Cdc42, Rac1, and RhoA

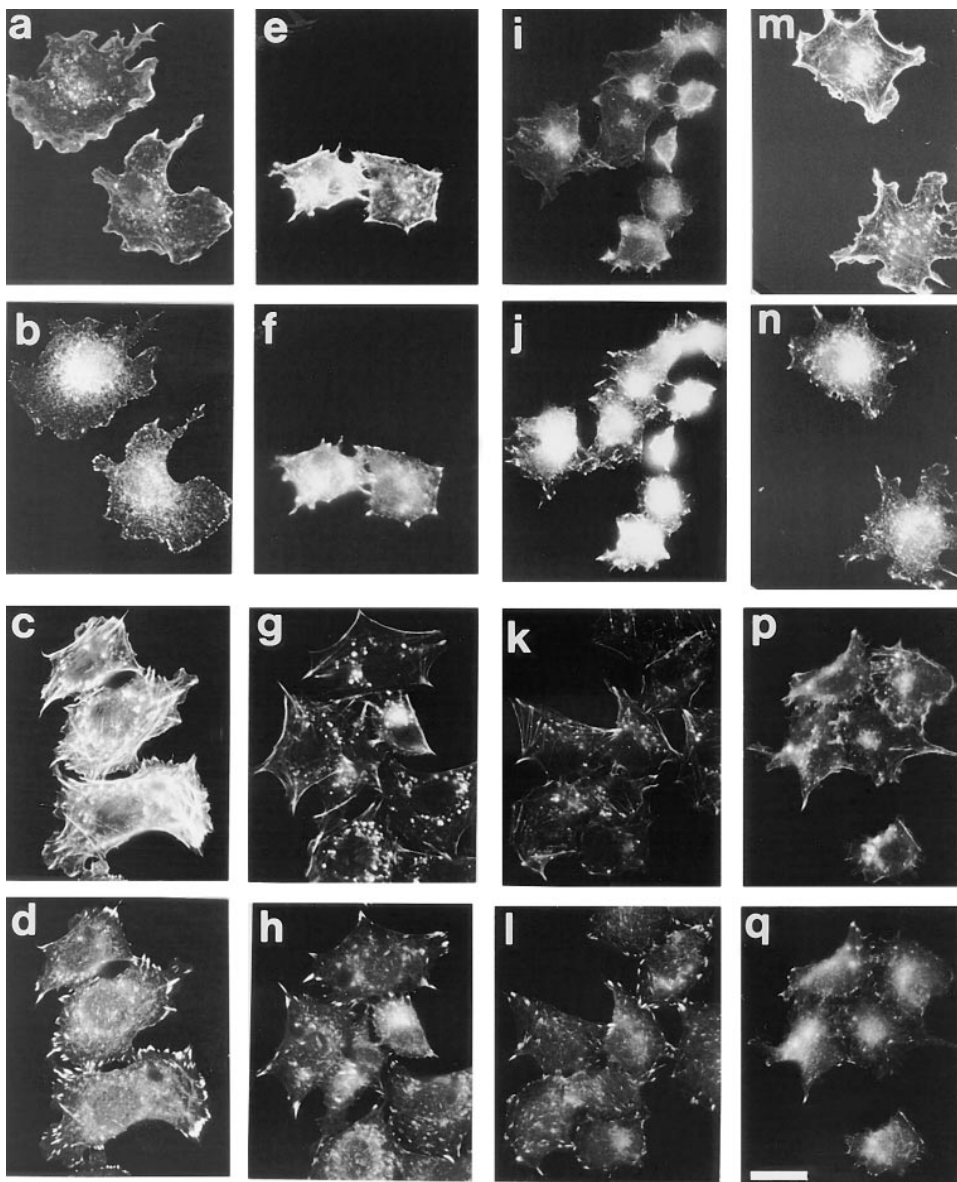


Figure 1. Assembly of focal adhesions and stress fibers during adhesion and spreading of Rat1 fibroblasts on fibronectin. Serum-starved Rat1 fibroblasts (*a–d*) or cells expressing dominant-inhibitory Cdc42 (*e–h*), Rac1 (*I–L*), or RhoA (*m–q*) were trypsinized, washed, and then replated on fibronectin-coated coverslips for 15 (*a, b, e, f, i, j, m, and n*) or 60 (*c, d, g, h, k, l, p, and q*) min and stained for vinculin (*b, d, f, h, j, l, n, and q*) and for F-actin (*a, c, e, g, i, k, m, and p*). Bar, 10 μ m.

(Fig. 3). The Rat1 cell lines selected for this study expressed the highest levels of N17Cdc42, N17Rac, and N19Rho; they have been previously characterized (Qiu et al., 1995*a,b*; Qiu et al., 1997). The levels of expression of the dominant-inhibitory mutants were sufficient to (1) inhibit PDGF-induced membrane ruffling in N17Rac-expressing cells (Fig. 2 *d*), and (2) inhibit stress fiber formation induced by serum in N19Rho-expressing cells (Fig. 2 *e*).

We examined if the Rho-family members Cdc42, Rac, and/or Rho control a cell's ability to adhere to and/or spread on a matrix. Rat1 cells expressing dominant-inhibitory mutants of Cdc42, Rac, or Rho were plated onto fibronectin for 20 min, fixed, stained, and then the number of spread and nonspread cells counted. No significant differences between Rat1 cells and dominant-inhibitory Cdc42-, Rac-, or Rho-expressing cells were detected in the numbers of cells that adhered to fibronectin (data not shown). However, a significant reduction in cell spreading was ob-

served in cells expressing dominant-inhibitory Cdc42, Rac, or Rho (Fig. 4).

A Role for Cdc42, Rac, and Rho in the Assembly of Focal Adhesions and Actin Fibers

To determine the roles of Rho family members in integrin-mediated cell morphology, Rat1 cells expressing dominant-inhibitory mutants of Cdc42, Rac, and Rho were plated onto fibronectin for 15 or 60 min and stained for F-actin. At the earliest time point examined (15 min), F-actin-containing ruffles were reduced in the N17Cdc42- and N17Rac-expressing cells (Fig. 1, *e* and *i*) when compared with the Rat1 control cells (Fig. 1 *a*). In contrast, membrane ruffles were prominent in N19Rho-expressing cells (Fig. 1 *m*), suggesting that both Cdc42 and Rac (but not Rho) are required for the adhesion-mediated organization of F-actin in membrane ruffles. Subsequent to

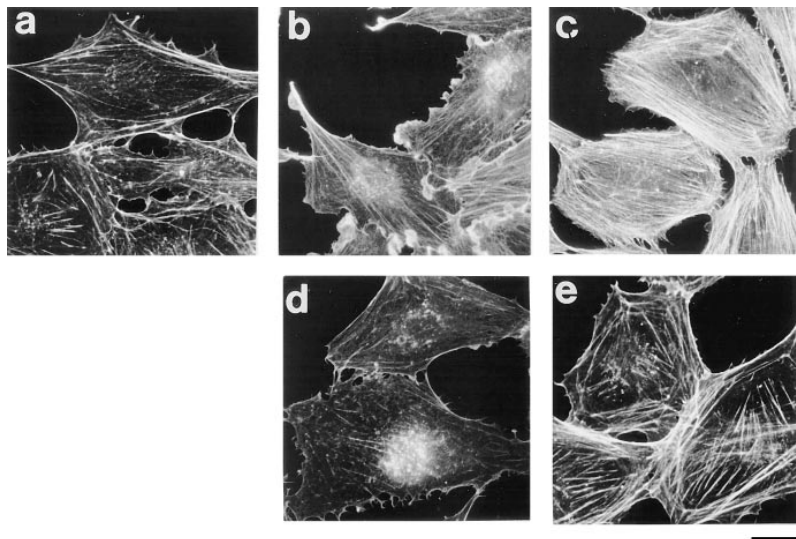


Figure 2. PDGF and serum induce the assembly of membrane ruffles and stress fibers in serum-starved adherent Rat1 cells. Parental (*a–c*), dominant-inhibitory Rac- (*d*), or Rho-expressing (*e*) Rat1 cells were plated on fibronectin-coated coverslips in serum-free media for 48 h. The cells were then left untreated (*a*) or stimulated with PDGF-BB (*b* and *d*; 3 ng/ml) or FBS (1%, *c* and *e*) for 10 min at 37°C. The cells were then washed, fixed, permeabilized, and then F-actin present in stress fibers was stained with phalloidin. Bar, 10 μm.

membrane ruffling, F-actin was organized into bundles. The intensity of phalloidin-stained F-actin in stress fibers appeared to be reduced in the N17Cdc42- (Fig. 1 *g*) and N17Rac-expressing (Fig. 1 *k*) cells, and N19Rho expression completely blocked the assembly of these structures (Fig. 1 *p*). To quantitate the reduction in F-actin observed in Rat1 cells expressing dominant-inhibitory mutants of Cdc42, Rac, or Rho, the cells were plated on fibronectin for 15 or 60 min and the amount of labeled phalloidin bound to F-actin quantitated as described in Materials and Methods. In the cells expressing dominant-inhibitory mutants of Cdc42, Rac, and Rho, F-actin levels were reduced to <50% of the level in control Rat1 cells (Fig. 5), suggesting that Cdc42, Rac, and Rho all control the rise in F-actin associated with adherence to fibronectin.

In parallel with our observations on actin organization, we stained the fibronectin-adherent cells for vinculin-containing focal adhesions. In the N17Cdc42- and N17Rac-expressing cells at the 60-min time point (Fig. 1, *h* and *l*, respectively), the size and/or number of vinculin-containing focal adhesions appeared reduced relative to the Rat1 control cells (Fig. 1 *d*). In contrast, small focal complexes were observed in the N19Rho-expressing cells at 15 min (Fig. 1 *n*), but the staining was more dramatically reduced by 60 min so that only a few faint focal adhesions were observed (Fig. 1 *q*). These results suggest that Rho is essential for adhesion-dependent focal adhesion assembly, but that Cdc42 and Rac also play a role in the assembly of these structures.

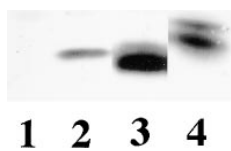


Figure 3. Expression of dominant-inhibitory mutants of Rho-family members Cdc42, Rac1, and RhoA in Rat1 cells. Rat1 fibroblasts (lane 1) or cells expressing a Myc epitope-tagged N17Cdc42 (lane 2), N17Rac1 (lane 3), or N19RhoA (lane 4) were grown for

48 h in the absence of 2 ng/ml tetracycline and the expression of these proteins was examined by immunoblotting with an antibody (9E10) to the Myc epitope.

To insure that the effects on cell morphology observed in cells expressing N17Cdc42, N17Rac, or N19Rho were not due to an adaptive response of the cells to the low levels of expression of the dominant-inhibitory mutants in uninduced cells, we performed transient transfections of N17Cdc42 or N17Rac1, or treated Rat1 cells with the Rho-specific inhibitor C3 transferase and monitored the adhesion-mediated organization of focal contacts and actin stress fibers. Cells expressing high levels of N17Cdc42 retained a rounded morphology 60 min after being plated onto fibronectin, and these cells had few (if any) detectable actin fibers (Fig. 6 *a*) or focal contacts (Fig. 6 *c*). Under the conditions of this experimental system, N17Cdc42 expression had a more pronounced effect on cell morphology (when compared with the stably expressing N17Cdc42 cell lines), perhaps resulting from the higher level of N17Cdc42 expression obtained (Fig. 6, *b* and *d*; and data not shown). Transient expression of N17Rac also inhibited the organization of actin fibers (Fig. 6 *e*) and focal contacts (Fig. 6 *g*), similar to what we observed in cells using the tetracycline-repressible system to express N17Rac. C3

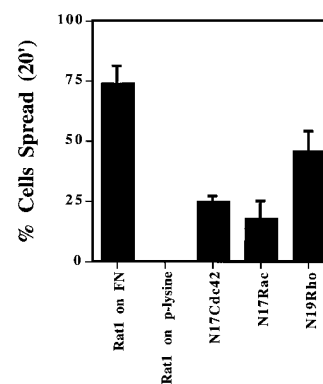


Figure 4. Rho, Rac, and Cdc42 are required for Rat1 cells to spread on fibronectin. Serum-starved parental (*Rat1*) or dominant-inhibitory Cdc42- (*N17Cdc42*), Rac1- (*N17Rac*), or RhoA-expressing (*N19Rho*) Rat1 fibroblasts were trypsinized, washed, and then replated on fibronectin- or polylysine-coated wells for 20 min. The cells were then fixed and stained with crystal violet. Random fields of cells were photographed and the percent cells spread was determined.

Error bars represent the standard deviation in triplicate samples. Similar results were obtained in three separate experiments. (Students' *t*-test: N17Cdc42, $P < 0.01$; N17Rac, $P < 0.02$; N19Rho, $P < 0.1$)

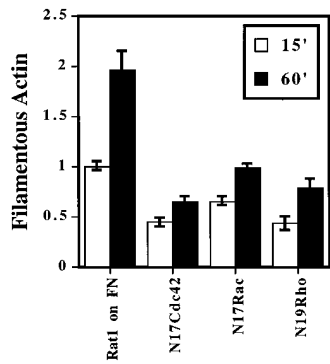


Figure 5. Quantitation of F-actin content in Rat1 cells adhering to fibronectin. Serum-starved parental (*Rat1*) or dominant-inhibitory Cdc42- (*N17Cdc42*), Rac1- (*N17Rac*), or RhoA-expressing (*N19Rho*) Rat1 fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 15 or 60 min. The cells were then fixed and phalloidin-stained, and the amount of phalloidin bound quantitated

as described in Materials and Methods. The relative F-actin content was determined by dividing the amount of phalloidin bound in fibronectin-adherent cells by the amount of phalloidin bound in fibronectin-adherent Rat1 cells at 15 min. Error bars represent the standard deviation in triplicate samples. Similar results were obtained in three separate experiments.

transferase inhibition of Rho blocked adhesion-induced stress fiber formation (Fig. 6 *i*) and focal adhesion organization (Fig. 6 *j*) in a manner similar to that observed in the N19Rho-expressing Rat1 cells (Fig. 1, *m* and *n*), with only peripheral point contacts and actin fibers detected. These results, obtained in transiently transfected or C3-treated Rat1 cells, serve to support the hypothesis stated above that Cdc42, Rac, and Rho control the assembly of focal adhesions and actin filaments; they suggest that Cdc42 and Rac control the generation of peripheral adhesion/cytoskeletal structures that are generated during cell spreading (an event that may indirectly control stress fiber formation), whereas Rho more directly controls the generation of focal adhesions and actin stress fibers.

Adhesion to Fibronectin Induces Integrin-mediated Signals Regulated by Rho and Cdc42

Not only do focal adhesions function as sites where structural components of a cell are assembled to regulate cell morphology, they also serve as a framework for the organization of signaling complexes that control integrin-mediated changes in cell behavior. Since the Rho family of small GTPases regulates several aspects of cell morphology as described above, we set out to identify integrin-mediated signaling events that are Cdc42, Rac, and/or Rho dependent. Since protein phosphorylation is one of the earliest events detected in response to integrin engagement (Guan et al., 1991; Kornberg et al., 1991), we chose to examine the role of Rho family GTPases in integrin-mediated tyrosine phosphorylation by observing: (*a*) the activation of several kinases (FAK, Src, Erk, and AKT) that are known to be activated by integrin stimulation; and (*b*) the tyrosine phosphorylation of some of the potential substrates of these kinases (FAK, Erk, and paxillin).

FAK, a tyrosine kinase that is activated and tyrosine phosphorylated in response to integrin–matrix interactions (Hanks et al., 1992; Kornberg et al., 1992; Lipfert et al., 1992), is also phosphorylated in response to agents that activate Rho (Zachary et al., 1992; Kumagai et al., 1993). This makes FAK a likely candidate to be regulated by the

Rho family of GTPases in response to adhesion. FAK tyrosine phosphorylation was monitored by immunoblotting FAK immunoprecipitates with a phosphotyrosine antibody (Fig. 7). FAK tyrosine phosphorylation in response to plating of Rat1 cells on fibronectin was unaffected in N17Cdc42- or N17Rac-expressing cells (<10%), but N19Rho expression inhibited the tyrosine phosphorylation of FAK by 50% ($n = 5$) at the 90-min time point (Fig. 7 *A*). Furthermore, when Rat1 cells were treated with C3 transferase to inhibit Rho activity, FAK tyrosine phosphorylation was inhibited by 40–60% ($n = 3$) (Fig. 7 *C*). Interestingly, we did not observe any inhibition of FAK tyrosine phosphorylation in the N19Rho-expressing cells at the 10-min time point (<5%, $n = 5$), suggesting that Rho may not be required for the earliest stages of FAK activation.

Paxillin is another focal adhesion protein that is tyrosine phosphorylated in response to integrin–matrix interactions (Burrige et al., 1992). In fact, it is a likely substrate for FAK. Paxillin tyrosine phosphorylation in response to adhesion of Rat1 cells to fibronectin was monitored by anti-phosphotyrosine immunoblotting of paxillin immunoprecipitates. Paxillin tyrosine phosphorylation was reduced by 70% ($n = 3$) in the N19Rho-expressing cells at the 90-min time point (Fig. 8 *A*) and 50% ($n = 2$) in C3 transferase-treated cells (Fig. 8 *C*), suggesting that the tyrosine phosphorylation of both FAK and paxillin is at least in part dependent on Rho.

The mitogen-activated protein (MAP) kinase Erk2 can be activated and phosphorylated downstream of FAK in an integrin-dependent manner (Chen et al., 1994; Schlaepfer et al., 1994). Since Erk2 activation is associated with a decrease in the electrophoretic mobility of the protein (or upward shift), we chose to examine the role of Rho family GTPases on Erk2 activity in response to plating of Rat1 cells on fibronectin by observing the mobility shift. Erk2 activation in Rat1 cells was rapid and transient, peaking at ~15 min, and then returning to near basal levels by 90 min (Fig. 9). All three dominant-inhibitory mutants (Fig. 9 *A*) and C3 transferase treatment (Fig. 9 *C*) diminished the activation of Erk2 to varying degrees (N17Cdc42 > C3 > N19Rho > N17Rac), with N17Cdc42 completely inhibiting integrin-mediated Erk2 activation and N17Rac having very little effect. Furthermore, adhesion-induced activation of Erk2 kinase activity, which was enhanced fourfold upon adhesion to fibronectin, was also inhibited when N17Cdc42 was transiently coexpressed (Fig. 9 *D*). Interestingly, N17Cdc42 expression did not inhibit Erk2 activation in PDGF-stimulated cells (Fig. 9 *B*), suggesting that Cdc42 may strictly be involved in integrin-mediated Erk2 activation.

Src is another protein tyrosine kinase activated upon integrin engagement that may play a role in signaling from the focal adhesion complex (Kaplan et al., 1995). To examine the role of Rho-family GTPases on Src activity in response to plating cells on fibronectin we immunoblotted detergent lysates with an antibody that specifically recognizes Src when it is phosphorylated at its autophosphorylation site (tyrosine 416) (Gao et al., 1997). Src was rapidly autophosphorylated in response to plating of Rat1 cells on fibronectin (Fig. 10). However, no significant difference (<15%) was observed in Src autophosphorylation in Rat1

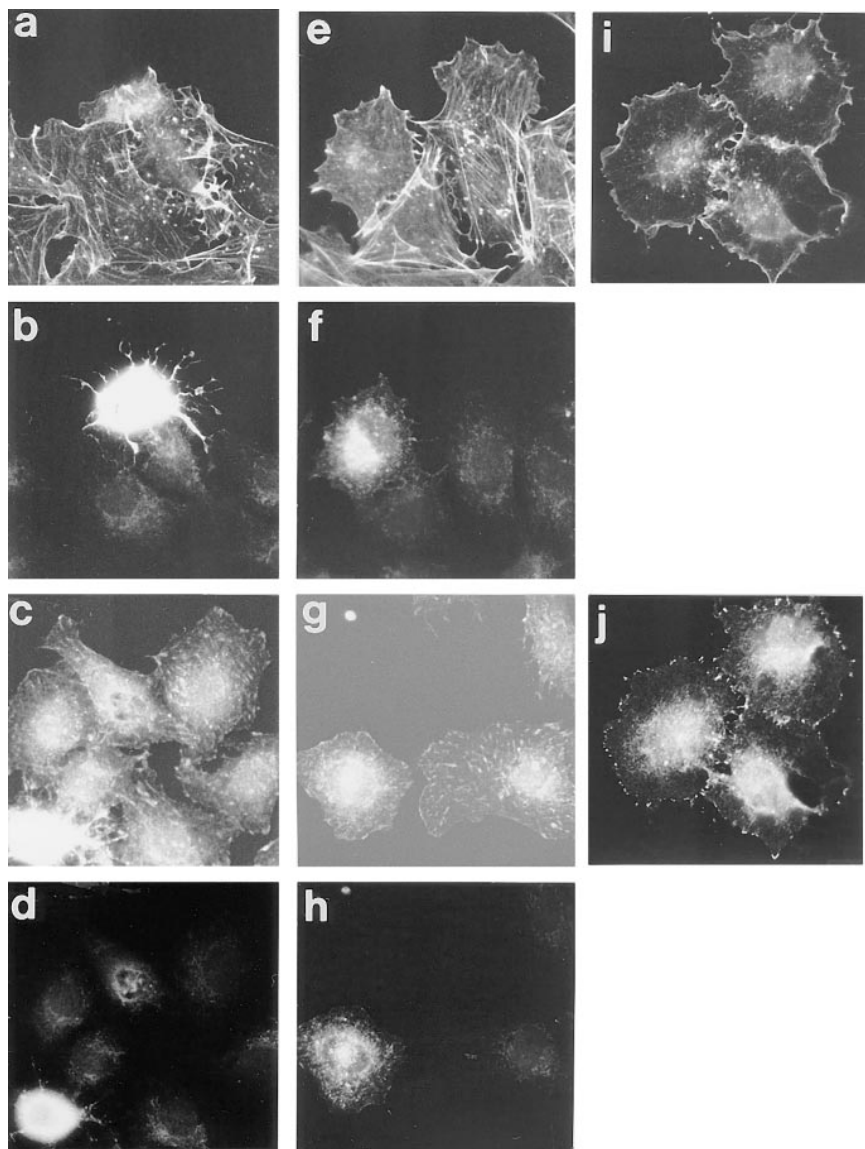


Figure 6. The role of Cdc42, Rac, and Rho in the assembly of focal adhesions and stress fibers in Rat1 fibroblasts. Rat1 cells transfected with 10 μ g of the pCMV plasmid encoding Myc-N17Cdc42 (*a-d*) or Myc-N17Rac1 (*e-h*) or Rat1 cells treated with C3 transferase (*i* and *j*) as described in Materials and Methods were trypsinized, washed, and then replated on fibronectin-coated coverslips for 60 min and stained for F-actin (*a*, *e*, and *i*), β 1 integrin (*c* and *g*), vinculin (*j*), or the Myc epitope tag (*b*, *d*, *f*, and *h*).

cells expressing dominant-inhibitory Rho family mutants, suggesting that integrin-mediated activation of Src is independent of Cdc42, Rac, and Rho.

Akt, an effector for phosphatidylinositol (PI) 3-kinase, is another kinase that can be activated upon attachment to fibronectin (King et al., 1997). We examined the potential role of Rho family GTPases in integrin-mediated Akt activation by performing immunoprecipitation/kinase assays using histone H2B as an exogenous substrate. Akt activity, which was activated threefold when Rat1 cells were plated on fibronectin, was unaffected by N17Rac or N19Rho expression but inhibited by 55% ($n = 3$) in N17Cdc42-expressing Rat1 cells (Fig. 11 *A*). The adhesion-induced Cdc42-dependent activation of Akt was further confirmed by transient co-transfection of N17Cdc42 and HA-tagged Akt in Rat1 cells. The twofold activation of Akt, observed after plating these cells on fibronectin for 15 and 60 min, was blocked by N17Cdc42 (Fig. 11 *B*). These data suggest that Cdc42 is necessary for integrin-dependent activation of Akt (and, by inference, PI 3-kinase).

Discussion

Numerous studies have examined how soluble extracellular factors induce the assembly of focal adhesions and stress fibers in serum-starved adherent Swiss 3T3 fibroblasts through activation of the Rho family of GTPases (Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997). In contrast, relatively little is known in detail about how these same actin-based structures are established de novo when cells adhere to the ECM via integrins. Therefore, the purpose of this study was to define the roles of the Rho family GTPases Cdc42, Rac, and Rho in (*a*) morphological changes that accompany cell adhesion and spreading on the ECM, and (*b*) regulating integrin-mediated signaling pathways.

Rho Family Members and Cell Morphology

We observed that inhibition of Cdc42, Rac, or Rho did not significantly affect Rat1 cell adhesion to fibronectin. Rho inhibition can block agonist-induced lymphocyte adhe-

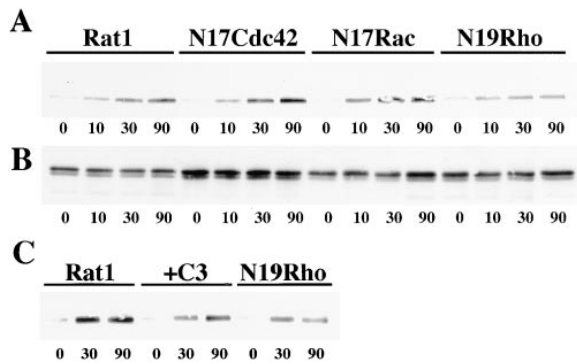


Figure 7. A role for Rho in integrin-mediated tyrosine phosphorylation of FAK. In *A* and *B*, serum-starved parental (*Rat1*) or dominant-inhibitory *Cdc42*- (*N17Cdc42*), *Rac1*- (*N17Rac*), or *RhoA*-expressing (*N19Rho*) *Rat1* fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 0, 10, 30, or 90 min. In *C*, *Rat1* cells treated with C3 transferase (+C3) as described in Materials and Methods were used and plated for 0, 30, or 90 min. The cells were then lysed, and 50 μ g of the lysates were immunoprecipitated with a polyclonal antiserum to FAK, subjected to electrophoresis, and then immunoblotted with an anti-phosphotyrosine antibody (*A* and *C*) or an anti-FAK antibody (*B*). The results presented here are representative of eight separate experiments.

sion, suggesting that Rho family members can control integrin-mediated cell adhesion in some (but not all) cell types (Laudanna et al., 1996). In contrast to cell adhesion, we found that the process of cell spreading is dependent on *Cdc42*, *Rac*, and *Rho*. This observation is in agreement with Barry et al. (1997) who found that Swiss 3T3 cells treated with the C3 transferase were poorly spread, suggesting that *Rho* regulates spreading in these cells. However, *Rho* can function as a negative regulator of cell spreading in phorbol ester-treated monocytes (Aepfel-

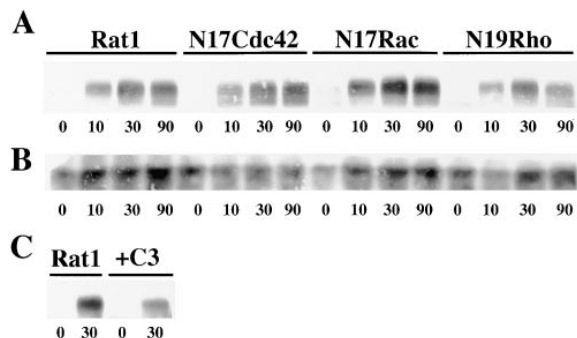


Figure 8. A role for Rho and *Cdc42* in integrin-mediated tyrosine phosphorylation of paxillin. In *A* and *B*, serum-starved parental (*Rat1*) or dominant-inhibitory *Cdc42*- (*N17Cdc42*), *Rac1*- (*N17Rac*), or *RhoA*-expressing (*N19Rho*) *Rat1* fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 0, 10, 30, or 90 min. In *C*, untreated *Rat1* cells (-) or *Rat1* cells treated with C3 transferase (+C3) as described in Materials and Methods were used and plated for 0 or 30 min. The cells were then lysed, and 100 μ g of the lysates were immunoprecipitated with a mAb to paxillin, subjected to electrophoresis, and then immunoblotted with an anti-phosphotyrosine antibody (*A* and *C*) or the anti-paxillin antibody (*B*).

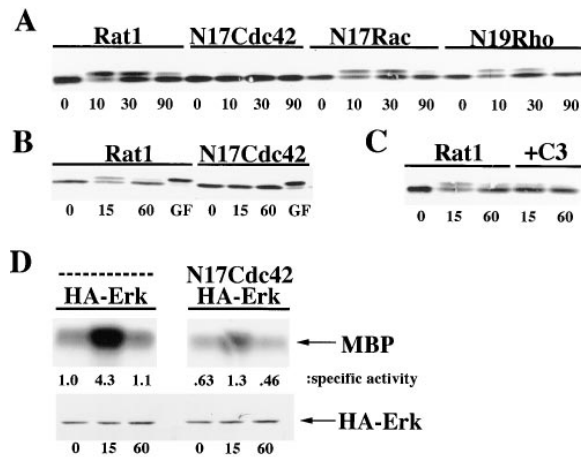


Figure 9. A role for Rho and *Cdc42* in integrin-mediated tyrosine phosphorylation of Erk2. In *A*, serum-starved parental (*Rat1*) or dominant-inhibitory *Cdc42*- (*N17Cdc42*), *Rac1*- (*N17Rac*), or *RhoA*-expressing (*N19Rho*) *Rat1* fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 0, 10, 30, or 90 min. In *B*, *Rat1* or *N17Cdc42*-expressing cells were replated on fibronectin for 0, 15, or 60 min, or treated in suspension with 10 ng/ml PDGF for 5 min at room temperature. In *C*, *Rat1* cells treated with C3 transferase (+C3) as described in Materials and Methods were used and plated for 0, 15, or 60 min. The cells were then lysed, and 15 μ g of the lysates were subjected to electrophoresis and immunoblotted with a pan-Erk antibody. In *D*, *Rat1* cells were transfected with 1 μ g of the pSVL plasmid encoding HA-ERK alone or in combination with 10 μ g of pCMV encoding Myc-*N17Cdc42*. The cells were serum-starved, trypsinized, washed, and then replated on fibronectin-coated dishes for 0, 15, or 60 min. HA-ERK was immunoprecipitated from cell lysates with the 12CA5 antibody and subjected to an in vitro kinase assay using myelin basic protein (*MBP*) as the substrate. HA-ERK present in the cell lysates (40 μ g) was detected by Western blotting with the 12CA5 antibody. The specific activity of HA-ERK is expressed relative to the specific activity of the kinase in cells transfected with HA-ERK alone and held in suspension.

bacher et al., 1996) or in thrombin-stimulated neuronal cells (Jalink et al., 1994), suggesting that *Rho* may positively or negatively regulate the process of cell spreading in different cell types.

During cell spreading on fibronectin, a series of morphological changes transpires: first, *Rat1* cells assemble actin-containing projections, membranous ruffles, and punctate vinculin-containing point contacts or focal complexes at the cell periphery; second, the focal complexes are replaced by full-blown focal adhesions from which bundles of F-actin radiate; and finally, there is an enhancement of F-actin-rich structures as the cells continue to spread on fibronectin. Our results suggest that most of these structures are regulated by *Rho* family GTPases but that different aspects require different GTPases (see Fig. 12 *A*). As it happens, the differing effects of the different dominant-inhibitory mutants provide evidence for the selectivity of these reagents.

N19Rho expression inhibits the matrix-induced assembly of focal adhesions and actin stress fibers, as has been observed previously (Hotchin and Hall, 1995; Barry et al., 1997). However, we found that, early in the process of cell



Figure 10. Integrin-mediated tyrosine phosphorylation of Src is independent of Cdc42, Rac, and Rho. Serum-starved parental (*Rat1*) or dominant-inhibitory Cdc42- (*N17Cdc42*), Rac1- (*N17Rac*), or RhoA-expressing (*N19Rho*) Rat1 fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 0, 10, 30, or 90 min. The cells were then lysed, and 15 μ g of the lysates were subjected to electrophoresis and immunoblotted with an antibody specific for Src phosphotyrosine-416 (Y416P) (A) or for total Src protein (B).

spreading, the small, vinculin-containing focal complexes that form do so in a Rho-independent manner. However, Rho appears to be required for the clustering of these complexes into larger focal adhesions. This hypothesis is supported by the observation that integrin cross-linking (which may occur when cells adhere to fibronectin) can induce Rho clustering at these sites (Burbelo et al., 1995) as

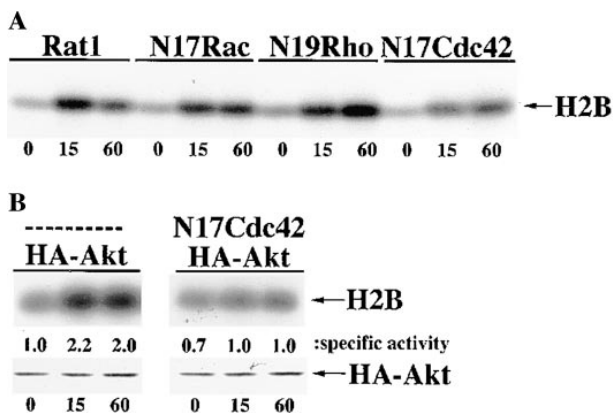


Figure 11. Integrin-mediated activation of Akt is Cdc42-dependent. Serum-starved parental (*Rat1*) or dominant-inhibitory Cdc42- (*N17Cdc42*), Rac1- (*N17Rac*), or RhoA-expressing (*N19Rho*) Rat1 fibroblasts were trypsinized, washed, and then replated on fibronectin-coated wells for 0, 15, or 60 min. Akt kinase activity was assayed using histone H2B as a substrate as described in Materials and Methods. This autoradiogram is representative of results from three separate experiments. In B, Rat1 cells transfected with 1 μ g of the pCMV-6 plasmid encoding HA-Akt either alone or in combination with 10 μ g of pCMV-encoding Myc-N17Cdc42 were serum starved, trypsinized, washed, and then replated on fibronectin-coated dishes for 0, 15, or 60 min. HA-Akt was immunoprecipitated with an antibody (12CA5) to the HA epitope and subjected to an in vitro kinase assay using histone H2B as the substrate. The samples were then electrophoresed and transferred to a nitrocellulose membrane for autoradiography (H2B) and quantitated on a PhosphorImager. The membrane was then probed with 12CA5 to determine the levels of HA-Akt present in the immunoprecipitate. The specific activity of HA-Akt (activity divided by protein levels) is expressed relative to the specific activity of the kinase in cells transfected with HA-Akt alone and held in suspension.

A

	Cell Spreading	F-Actin in Ruffles	F-Actin in Stress Fibers	Focal Contact Assembly	FAK Tyrosine Phosphorylation	Paxillin Tyrosine Phosphorylation	Erk2 Phosphorylation	Src Tyrosine Phosphorylation	Akt Activation
N17Cdc42	↓	↓	↓	↓	—	↓	↓	—	↓
N17Rac	↓	↓	↓	↓	—	—	↓	—	—
N17Rho or C3	↓	—	↓	↓	↓	↓	↓	—	—

↓ Complete inhibition ↓ Partial inhibition ↓ Slight inhibition — No inhibition

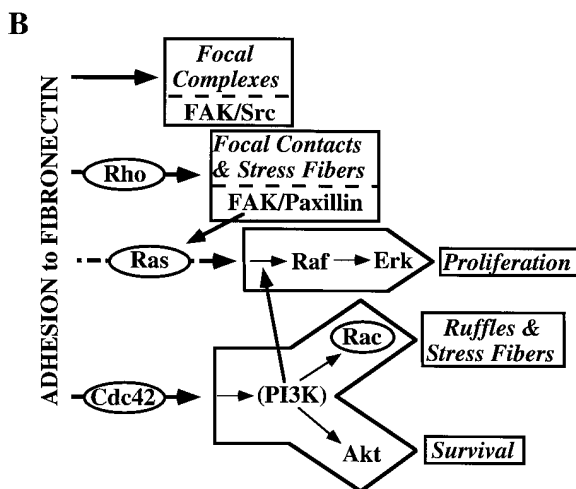


Figure 12. Summary of the different roles of Rho-family GTPases, Cdc42, Rac, and Rho in regulating cell morphology and integrin-mediated signaling events. The ability of N17Cdc42, N17Rac, N19Rho, or C3 transferase to inhibit completely (>90%, large arrow), partially inhibit (40–70%, small arrow), inhibit at a single time point (white arrow), or not significantly inhibit (<10%, dash) integrin-mediated morphological changes and signaling events that occur upon adhesion to fibronectin are summarized in A. B shows a model of the different roles played by Rho family GTPases in integrin-mediated signaling events that regulate cytoskeletal organization. Cell adhesion to the extracellular matrix induces the aggregation of small clusters of integrin receptors that leads to cytoskeletal organization via several pathways. Initial activation of FAK and Src, and the assembly of focal complexes are independent of Rho family members. A Rho-dependent pathway regulates the full activation of FAK, phosphorylation of paxillin, and assembly of focal adhesions. A third pathway from clustered integrins activates Akt (probably through PI 3-kinase) in a Cdc42-dependent pathway. Both Cdc42 and Rac regulate actin organization required for membrane ruffles. Adhesion-dependent Erk phosphorylation is contingent upon the activity of Cdc42 and Rho, and it requires a properly organized cytoskeleton. Rho, Cdc42, and Rac all regulate the organization of F-actin in stress fibers, although it is unlikely that they do so through a single, linear “cascade” since some events, such as FAK phosphorylation, are regulated by only a single Rho family member. Therefore, the most plausible explanation is that Rho family members regulate adhesion-initiated stress fiber formation via convergent pathways.

a precursor to the Rho-dependent generation of cell contraction that stimulates the aggregation of integrins into larger focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Taken together, these results suggest that when suspended cells are plated on ECM substrates, they first assemble small focal complexes that subsequently cluster into larger focal adhesions in a Rho-dependent manner; this latter stage may be analogous to the assembly of focal adhesions in serum-starved adherent cells stimulated with Rho-activating agents such as lysophosphatidic acid (LPA) (Ridley and Hall, 1992). Expression of moderate levels of N17Cdc42 or N17Rac in Rat1 cells had a modest effect on the generation of focal adhesions, causing an apparent delay in the assembly of the larger focal adhesions. However, transient overexpression of N17Cdc42 had a more dramatic effect on the generation of focal adhesions than that observed in the cells expressing N17Cdc42 stably. Taken together, these results suggest that Rho is the primary small GTPase regulating focal adhesions, but Cdc42 may also play a prominent role.

In contrast to focal adhesion assembly, we observed that the generation of microfilament bundles when Rat1 cells adhere to fibronectin is regulated by all three small GTPases. At first glance, this observation would appear to suggest that focal adhesions can be generated without prior assembly of actin stress fibers, a conclusion supported by a previous study in which Rho-induced focal adhesion assembly occurred in the presence of inhibitors of actin filament assembly (Nobes and Hall, 1995). However, it should be noted that whereas the actin bundles observed in cells expressing N17Cdc42, N17Rac, and N19Rho were much less intense than those observed in Rat1 control cells, every vinculin-containing focal adhesion appears to be associated with a bundle of actin filaments. This observation is consistent with the idea that focal adhesions form as a result of the contractility of stress fibers (Chrzanowska-Wodnicka and Burridge, 1996).

The precise mechanism whereby Rho family GTPases regulate the assembly of the F-actin component of actin stress fibers has been the subject of numerous studies. It has been suggested that Rho regulates stress fiber formation in mitogen-stimulated Swiss 3T3 cells by inducing the bundling of existing F-actin into larger actin stress fibers and not by inducing actin polymerization (Machesky and Hall, 1997); we have found, using a different assay, that Rho is required for an adhesion-induced increase in F-actin. The apparent discrepancy is likely to be the result of differences in the mechanisms by which insoluble (matrix) and soluble factors stimulate actin polymerization, as has been previously noted in other cell types (Southwick et al., 1989). It is also possible that in our assay we are measuring a Rho-dependent stabilization of F-actin rather than de novo actin polymerization. We have also observed that Rac is required for adhesion-induced actin polymerization; this finding is in keeping with observations that Rac (but not Rho) is essential for the uncapping of actin filaments (Hartwig et al., 1995), and subsequent actin monomer incorporation into stress fibers and lamellipodia (Machesky and Hall, 1997). Furthermore, it was recently observed that Cdc42 (but not Rho or Rac) regulates actin polymerization in a cell-free system (Zigmond et al., 1997; Ma et al., 1998), and we observed that N17Cdc42 expres-

sion inhibited the adhesion-induced increase in F-actin. Finally, genetic evidence suggests that both Rac and Cdc42 control actin-dependent processes; Rac controls actin assembly at adherens junctions and Cdc42 controls actin-dependent epithelial cell polarization in *Drosophila* (Eaton et al., 1995). Taken together, these results suggest that Rho, Rac, and Cdc42 are all capable of regulating F-actin content within a cell and that all three do so upon cell adherence. The possible mechanisms whereby Rho family GTPases regulate integrin-dependent actin organization will be discussed below.

Rho Family Members in Integrin-mediated Signaling

Numerous signal transduction molecules associate with integrin complexes in adherent cells, placing them in an ideal location to respond to and regulate adhesion-dependent changes in cell morphology (Yamada and Miyamoto, 1995). One of the earliest responses to integrin-dependent adhesion is tyrosine phosphorylation (Guan et al., 1991; Kornberg et al., 1991), a response that is also elicited by LPA in a Rho-dependent manner (Barry and Critchley, 1994; Ridley and Hall, 1994; Flinn and Ridley, 1996). These results suggest that Rho could function downstream of integrins in directing adhesion-dependent tyrosine phosphorylation. Tyrosine kinase(s) may also function upstream of Rho since the tyrosine kinase inhibitor tyrphostin A25, which blocks the assembly of LPA-induced focal adhesions, can be overcome by microinjection of activated Rho (Nobes et al., 1995). Therefore, protein tyrosine kinases appear to play a role both upstream and downstream of Rho in integrin-mediated tyrosine phosphorylation.

There are several candidate kinases that may be responsible for integrin-dependent tyrosine phosphorylation, most notably FAK and Src. FAK is tyrosine phosphorylated and activated in response to integrin stimulation or upon LPA stimulation, and LPA-mediated FAK phosphorylation is Rho dependent (Hanks et al., 1992; Kornberg et al., 1992; Lipfert et al., 1992; Kumagai et al., 1993; Seckl et al., 1995). We found that Rho, but not Rac or Cdc42, is required for the complete phosphorylation of FAK induced by matrix adhesion; this result is in agreement with Barry et al. (1997), suggesting that FAK activation is in part Rho dependent. However, we observed that at the earliest times examined FAK phosphorylation was not inhibited by N19Rho expression, suggesting that there is a Rho-independent component to integrin-dependent FAK phosphorylation. This observation is supported by data from Miyamoto et al. (1995) showing that aggregation of integrins can cause FAK to be phosphorylated without the accumulation of Rho-dependent F-actin. Furthermore, since there are multiple sites on which FAK is phosphorylated, it is possible that Rho may regulate some but not all of these sites. One of these sites is tyrosine 397 that, when phosphorylated, creates a docking site for the Src homology 2 (SH2) domain when cells adhere to fibronectin (Schlaepfer et al., 1994). Since the Src SH2 domain normally interacts with the phosphorylated tyrosine 527 at Src's COOH terminus, an interaction that maintains Src in a low activity state (Parson and Parsons, 1997), association of Src with FAK may occur concomitantly with Src activation. Src is activated in an integrin-dependent manner after

cell adhesion (Kaplan et al., 1995). We found that plating of Rat1 cells on fibronectin induced the phosphorylation of Src at tyrosine 416, the autophosphorylation site that is phosphorylated in activated forms of Src. The phosphorylation of Src was not dependent on Cdc42, Rac, or Rho, suggesting that Src is unlikely to be one of the kinases downstream of Rho family GTPases.

After integrin-dependent assembly of the FAK–Src complex, additional signaling elements may then be recruited to link adhesion to activation of the Ras/MAP kinase pathway (Chen et al., 1994; Schlaepfer et al., 1994; Clark and Hynes, 1996). Integrin-dependent activation of the MAP kinase Erk2 is partially inhibited by N19Rho expression or C3 transferase treatment, suggesting that Rho plays a role in the activation of this kinase (Renshaw et al., 1996). We found that in addition to Rho playing a role in Erk2 activity, Cdc42 also regulates Erk2 activation since N17Cdc42 expression almost completely inhibited Erk2 phosphorylation and kinase activity. All three Rho family GTPases may control Erk2 activity through their effects on the cytoskeleton, since treatment of cells with cytochalasin D inhibits adhesion-induced (but not growth factor-induced) Erk2 activity (Chen et al., 1994). Interestingly, N17Cdc42 expression did not affect PDGF-induced Erk2 activation, suggesting that growth factor activation of Erk2 is Cdc42 independent. This result is in contrast to our earlier observation that Ras controls both integrin-dependent and growth factor-induced activation of Erk2 (Clark and Hynes, 1996), which suggests that Ras and Cdc42 control integrin-dependent Erk2 activation by distinct mechanisms. Cdc42 regulation of adhesion-dependent Erk2 activation may play a role in the control of anchorage-independent growth by Cdc42 (Qiu et al., 1997).

The cytoskeletal protein paxillin is another potential effector for the FAK–Src complex. Paxillin colocalizes with FAK to focal adhesions, can physically associate with FAK, and is tyrosine phosphorylated in response to adhesion to fibronectin (Burrige et al., 1992; Clark and Brugge, 1995). In addition, overexpression of a FAK mutant that is incapable of binding to Src fails to induce paxillin phosphorylation, and expression of the COOH-terminal domain of FAK inhibits both FAK activation and paxillin tyrosine phosphorylation (Schaller and Parsons, 1995; Richardson and Parsons, 1996). Paxillin could be a downstream mediator of signals initiated by FAK, since paxillin tyrosine phosphorylation, like that of FAK, is at least partially Rho-dependent (Rankin et al., 1994; Barry et al., 1997). It has been suggested that the tyrosine phosphorylation of paxillin may recruit SH2-containing proteins essential for focal adhesion formation to sites of adhesion (Turner, 1993). However, while FAK inhibition blocks adhesion-associated tyrosine phosphorylation, it does not inhibit focal adhesion assembly (Gilmore and Romer, 1996). Our finding that paxillin tyrosine phosphorylation does not proceed in N19Rho-expressing cells that fail to assemble focal adhesions is consistent with the hypothesis that Rho-dependent focal adhesion formation regulates paxillin tyrosine phosphorylation (Burrige and Chrzanowska-Wodnicka, 1996).

The serine-threonine kinase Akt (also known as PKB), a close relative of protein kinase C, has been shown to be a target of PI 3-kinase (Datta et al., 1996; Franke et al.,

1997). Integrin-dependent activation of Akt also requires PI 3-kinase (King et al., 1997). Our finding that integrin-dependent activation of Akt is suppressed when N17Cdc42-expressing Rat1 cells are plated on fibronectin therefore suggests that Cdc42 lies upstream of PI 3-kinase and Akt in an integrin-mediated signaling pathway. This result is consistent with several observations: first, that Cdc42 binds the regulatory subunit of PI 3-kinase, stimulating PI 3-kinase activity (Zheng et al., 1994); and second, that Cdc42 induces integrin-mediated cell motility through a PI 3-kinase-dependent pathway (Keely et al., 1997). In addition, integrin-mediated PI 3-kinase activity is sufficient to stimulate Rac-dependent ruffling (Reif et al., 1996; Rodriguez-Viciana et al., 1997). We therefore propose an integrin-mediated signaling pathway that includes Cdc42-dependent activation of PI 3-kinase, PI 3-kinase-dependent activation of Rac, and Rac-dependent generation of membrane ruffles (see Fig. 12 B). This pathway may also regulate integrin-dependent Erk2 phosphorylation, since both Cdc42 (Fig. 9) and PI 3-kinase (King et al., 1997) contribute to Erk2 phosphorylation that occurs when cells adhere to fibronectin.

The Role of Rho Family GTPases in Integrin-mediated Cytoskeletal Organization

We have provided evidence that, like soluble factors, the ECM controls cytoskeletal organization through several distinct signal transduction pathways regulated by different members of the Rho family of GTPases (see Fig. 12 B). When cells initially adhere to the ECM, punctate focal complexes form at the cell periphery. These clusters of adhesion molecules are capable of initiating some signaling involving FAK and Src independent of Rho family GTPases (Fig. 12 B, *top*). However, subsequent FAK activation occurs somewhat more slowly in a Rho-dependent manner. This Rho-dependent pathway also controls paxillin phosphorylation and the assembly of large focal adhesions from which actin stress fibers radiate. Initial adhesion also stimulates peripheral F-actin staining in membrane ruffles. This actin organization is independent of Rho but is dependent on both Cdc42 and Rac (Fig. 12 B, *bottom*). Since Cdc42 controls the integrin-dependent activation of Akt (whose activation is PI 3-kinase-dependent; King et al., 1997), and since PI 3-kinase can stimulate Rac-dependent membrane ruffling, Cdc42, PI 3-kinase, and Rac could lie on the same pathway that regulates adhesion-induced membrane ruffling. Finally, significant cross-talk between these pathways is likely: the Cdc42- and Rho-dependent pathways may regulate cell proliferation via the Ras/Raf pathway (Schlaepfer et al., 1994; King et al., 1997).

The pathways defined in the model we present for adhesion-induced signaling (Fig. 12 B) are the result of combining the results obtained in this study with several recent observations (Clark and Hynes, 1996; King et al., 1997; Qiu et al., 1997; Rodriguez-Viciana et al., 1997). Our model, in which Ras, Rac, Rho, and Cdc42 play roles in parallel, convergent pathways, can be contrasted to the “cascade” model for regulation of Rho family GTPases that has solidly established itself as a paradigm in the field (Chant and Stowers, 1995). This model is based, in large part, on microinjection studies in serum-starved Swiss 3T3

fibroblasts that have indicated that Rho family GTPases can function in a cascade-like fashion to organize the actin cytoskeleton with Cdc42 activating Rac, which activates Rho (Nobes and Hall, 1995). However, other studies in Swiss 3T3 fibroblasts have shown that constitutively active Cdc42 causes stress fiber disassembly (Kozma et al., 1995). In addition, in neuronal cells an antagonistic relationship has been noted between Rho on the one hand, and Cdc42 and Rac on the other (Kozma et al., 1997). Finally, our results detailing the regulation of FAK and paxillin tyrosine phosphorylation by Rho, but not by Rac or Cdc42, suggest that these GTPases are not controlled in a simple linear cascade (from Cdc42 to Rac to Rho) during cell adhesion and spreading on fibronectin. Instead, Cdc42- and Rac-dependent pathways converge with Rho-dependent pathways to control cell morphology (see Fig. 12 B). Taken together, these results suggest that alternatives to the cascade model may describe the signaling pathways regulated by Rho family GTPases in other systems (e.g., adhesion-based signaling).

These independent pathways are envisaged as converging, through their effects on focal adhesion assembly and/or actin microfilament organization, to give order to the actin-based cytoskeleton. While this skeleton plays a vital role in cell morphology and movement, it can also affect MAP kinase signaling pathways that regulate gene expression and cell proliferation (Schwartz et al., 1995). How might Cdc42, Rac, and Rho regulate the integrin-mediated organization of this actin-based cytoskeleton? Cdc42 and Rac may do so through the serine/threonine kinase Pak1 (p21-activated kinase), since activated Pak1 is capable of inducing filopodia and ruffles (Sells et al., 1997). However, Cdc42- and Rac-effector mutants that do not interact with Pak1 still induce cytoskeletal changes (Joneson et al., 1996; Lamarche et al., 1996), suggesting that Cdc42 and Rac may use several pathways to regulate actin organization. Cdc42 may regulate the actin cytoskeleton through its association with the Wiskott-Aldrich Syndrome protein, a protein whose absence in affected individuals causes cytoskeletal abnormalities of T cells and platelets (Symons et al., 1996). Rac also stimulates the uncapping of actin filaments through a polyphosphoinositide (PPI)-dependent mechanism (Hartwig et al., 1995), suggesting that Rac may control cytoskeletal organization through regulation of PPI synthesis. Rho may also control the synthesis of cellular PPIs by regulating the phosphatidylinositol 4-phosphate (PIP) 5-kinase (Chong et al., 1994). These results suggest that Rac and/or Rho may regulate actin organization by controlling the cellular levels of PPIs in a Rac- or Rho-dependent manner. Further evidence that PIP₂ may play a role in cytoskeletal organization comes from studies on PIP 5-kinase suggesting that PIP₂ is capable of inducing actin polymerization in vivo (Shibasaki et al., 1997). PIP₂ may then regulate the organization of the cytoskeleton through regulation of vinculin binding to talin and actin (Gilmore and Burridge, 1996; Weekes et al., 1996). However, stimulation of actin polymerization in a cell-free system does not correlate with PIP₂ synthesis (Zigmond et al., 1997), indicating that additional work must be done in defining the role of PIs in regulating the actin cytoskeleton. Finally, several RhoA-binding kinases (ROK α , Rho-kinase) are capable of promoting the forma-

tion of focal adhesions and actin stress fibers when introduced into cells (Leung et al., 1996; Amano et al., 1997; Ishizaki et al., 1997). These Rho-binding kinases can phosphorylate the myosin-binding subunit of myosin light chain (MLC) phosphatase (thereby inactivating the phosphatase, resulting in MLC phosphorylation) and MLC itself (Amano et al., 1996; Kimura et al., 1996). Phosphorylated MLC induces a conformational change in myosin, increasing its binding to actin filaments and triggering the assembly of stress fibers (Citi and Kendrick-Jones, 1987; Burridge and Chrzanowska-Wodnicka, 1996). Rho kinase may also regulate the cytoskeleton by phosphorylating the ezrin/radixin/moesin proteins (Matsui et al., 1998; Shaw et al., 1998). These results suggest that Rho kinases may be important effectors for Rho in controlling the organization of the cytoskeleton.

In summary, we have shown that adhesive interactions stimulate the formation of punctate focal complexes at the cell periphery during cell spreading, and cell spreading is dependent on Cdc42, Rac, and Rho. Membrane ruffling occurs through a mechanism that is dependent on Cdc42 and Rac, whereas Rho controls the assembly of large focal adhesions from which actin stress fibers radiate. Rho also regulates the majority of FAK activation and the phosphorylation of paxillin, events that may modulate the organization of the cytoskeleton during the assembly of Rho-dependent focal adhesions, whereas Cdc42 regulates adhesion-associated Akt and Erk2 activation.

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