Rho GTPases Control Polarity, Protrusion, and Adhesion during Cell Movement

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Abstract. Cell movement is essential during embryogenesis to establish tissue patterns and to drive morphogenetic pathways and in the adult for tissue repair and to direct cells to sites of infection. Animal cells move by crawling and the driving force is derived primarily from the coordinated assembly and disassembly of actin filaments. The small GTPases, Rho, Rac, and Cdc42, regulate the organization of actin filaments and we have analyzed their contributions to the movement of primary embryo fibroblasts in an in vitro wound healing assay. Rac is essential for the protrusion of lamellipodia and for forward movement. Cdc42 is required to maintain cell polarity, which includes the localization of lamellipodial activity to the leading edge and the reorientation of the Golgi apparatus in the direction of movement. Rho is required to maintain cell adhesion during movement, but stress fibers and focal adhesions are not required. Finally, Ras regulates focal adhesion and stress fiber turnover and this is essential for cell movement. We conclude that the signal transduction pathways controlled by the four small GTPases, Rho, Rac, Cdc42, and Ras, cooperate to promote cell movement.

Key words: Rho GTPases • Ras • polarity • focal adhesion • wound healing

CELL migration is a key aspect of many normal and abnormal biological processes, including embryonic development, defense against infections, wound healing, and tumor cell metastasis (reviewed in Trinkaus, 1984; Martin, 1997). It is generally accepted that the driving force for cell movement is provided by the dynamic reorganization of the actin cytoskeleton, directing protrusion at the front of the cell and retraction at the rear. In addition, however, efficient migration requires coordinated changes in other cellular activities such as directed secretion (to provide new membrane components), turnover of cell–matrix interactions (to control adhesion), and changes in gene transcription (to provide autocrine/paracrine signals) (reviewed in Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996).

Polymerized actin in a migrating cell is organized into three major types of filamentous array, each linked to the underlying matrix via integrin receptors (Small et al., 1996). Lamellipodia, sheetlike structures consisting of a cross-linked meshwork of actin filaments, and filopodia, fingerlike structures consisting of thin parallel bundles of actin filaments, are two protrusive structures found at the cell's leading edge. Both form weak sites of attachment to the substrate called focal complexes (Nobes and Hall, 1995). Positioned back from the leading edge is a third class, actin–myosin filament bundles, most clearly seen in fibroblasts as stress fibers and dorsal arc-like arrays which insert into large focal adhesion complexes (Small et al., 1996). These contractile filaments are thought to provide the driving force for forward movement of the rear of the cell (Dunn, 1980; Chen, 1981).

The molecular control of actin filament assembly and disassembly is likely to underpin cell motility in all cell types and in this regard the Rho family of small GTPases, particularly Rho, Rac, and Cdc42, has received a lot of attention (reviewed in Ridley, 1996; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). In Swiss 3T3 fibroblasts, Rho regulates the formation of contractile actin–myosin filaments to form stress fibers (Ridley and Hall, 1992), while Rac and Cdc42 regulate lamellipodia and filopodia formation, respectively (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). Rho is also required for the formation and maintenance of focal adhesions (Ridley and Hall, 1992; Hotchin and Hall, 1995), whereas Rac and Cdc42 regulate the formation of smaller "focal complex" structures associated with lamellipodia and filopodia (Nobes and Hall, 1995). Furthermore, in fibroblasts the activities of these three proteins are linked to each other in a hierarchical fashion: Cdc42 can rapidly activate Rac, leading

to the intimate association of filopodia and lamellipodia, while Rac can activate Rho, leading potentially to the formation of new sites of focal adhesions and contractile filament assembly within an advancing lamellipodium (Nobes and Hall, 1995). Therefore, it is likely that the Rho GTPase family plays an important role in regulating cell movement.

Different kinds of cell movement have been observed both in the adult and in the embryo. In some cases, cells move as isolated individuals, for example leukocytes crawling towards chemotactic factors or neuroblasts migrating during development. In other cases, cells move coordinately, often as sheets, for example embryonic epithelial cells during dorsal closure or fibroblasts during adult wound healing (Gumbiner, 1996; Noselli, 1998). These different modes of cell movement are likely to involve some common and some distinct activities. To dissect the molecular mechanisms underlying cell migration, various tissue culture models have been used. A commonly used model is the Boyden chamber, which measures the migration of individual cells across a porous membrane towards a source of chemoattractant. However, a more precise observation of directed motility of individual cells can be achieved using a Dunn chemotaxis chamber (Allen et al., 1998). We have made use of a wound healing assay, using a monolayer of primary rat embryo fibroblasts (REFs),[1] to analyze mechanisms underlying coordinated cell movement. This model results in essentially unidirectional and synchronous movement of cells and has allowed us to analyze the role of small GTPases in regulating distinct parameters of cell migration.

Materials and Methods

Materials

LY294002 (Calbiochem) was prepared as a stock solution (1,000×) of 20 mM in DMSO and frozen at −20°C. PD98059, 2-(2′-amino-3′methoxyflavone), was purchased from Calbiochem and a stock solution (30 mM; 500×) was prepared in DMSO and frozen at −20°C. The effectiveness of PD98059 was confirmed by its ability to inhibit wound-induced phosphoERK activation. Cycloheximide was prepared as a stock solution (1,000×) of 100 mg/ml in DMSO. The myosin inhibitor 2,3-butanedione monoxime (BDM; Sigma Chemical Co.) was prepared as a stock solution of 0.5 M in H_2O and was added to a final concentration of 15 mM and was readded after 3 h. The effectiveness of BDM was assessed by its ability to disrupt the organization of myosin II in stress fibers. The p160ROCK inhibitor, Y-27632 (Uehata et al., 1997; Hirose et al., 1998), was prepared as a stock solution of 3 mM in DMSO. The effectiveness of Y-27632 was assessed by its ability to disrupt actin stress fibers and focal adhesions.

Cell Culture

Primary REF cells were provided by Dr. Durward Lawson (University College London, London, UK). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, streptomycin, and penicillin at 37°C in 10% CO_2. Wound-induced cell migration assays were performed on secondary cells between passages 3 and 7.

Proteins, Antibodies, and Reagents

Recombinant V14Rho (RhoA isotype), V12Rac (Rac1 isotype), V12-Cdc42 (G25K isotype), C3 transferase, N17Rac, N17Cdc42, and WASp (Cdc42 binding fragment comprising amino acids 201–321) were expressed as glutathione S-transferase fusion proteins in *Escherichia coli*

and purified on glutathione-agarose beads essentially as described in Self and Hall (1995). The proteins were released from the beads by thrombin cleavage and dialyzed against microinjection buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$, 0.1 mM DTT) and concentrated as required. For the GTP-binding proteins, active protein concentrations were determined by filter binding assay using [^{3}H]GDP of [^{3}H]GTP as described previously (Self and Hall, 1995). N17Cdc42 has a low affinity for [^{3}H]GDP (Self and Hall, 1995) and therefore the protein concentration for N17Cdc42 was estimated using a protein assay kit (Bio-Rad Laboratories). The protein concentrations of C3 transferase and the WASp fragment were also determined by this method. Purified neutralizing antibody to Ras, Y13-259, was a kind gift of Dr. Hugh Paterson (Chester Beatty Laboratories, London, UK).

Wounding, Microinjection, and Inhibitor Treatments

REFs for wound assays were seeded at a high density, 12×10^4 cells, on 13-mm glass coverslips, and wounded 1 d later when the cells formed a confluent monolayer. The wound was made by scraping a microinjection needle (broken to its shaft and flame polished) through/across the cell monolayer. The wound width was consistently between 100 and 130 μm and wounds reproducibly took between 5 and 6 h to close. Cells were pretreated with inhibitors for 20 min or, in the case of Y-27632, 1 h before wounding. Since most wound edge cells round up immediately upon wounding and thus are difficult to inject, wounds were left for 1 h to allow cell respreading and to facilitate microinjection. Proteins were injected into the cell cytoplasm along with a marker protein (either FITC- or Texas red–conjugated, lysinated dextrans at 2 mg/ml). Recombinant proteins were microinjected at concentrations as indicated in the text. The neutralizing anti-Ras antibody, Y13-259, was microinjected at a concentration of 8–9 mg/ml. Expression vectors (pRK5-myc) encoding N17Rac1, N17Cdc42 (G25K isoform), WASp fragment, and V12HaRas were injected into the cell nucleus at a concentration of 200 μg/ml in PBSA and expressed protein was visualized using anti-myc antibodies (9E10) or in the case of Ras with the rat monoclonal antibody, Y13-238. Previously we have shown that at least 90% of DNA-injected cells express the pRK5 construct (Lamarche et al., 1996) and myc-tagged protein could be detected by 30 min after cell injection.

Immunofluorescence Staining Protocols

For each experimental run, control wounds were fixed soon after the wound was made (for inhibitor experiments), 1 h after wounding (for microinjection experiments) and immediately after the wound edges have met as monitored by frequent observation using phase-contrast optics. Experimental wounds were fixed at the time that control wounds had closed. For wound closure measurements, cells were stained for filamentous actin as previously described (Nobes and Hall, 1995). In brief, cells were fixed in 4% paraformaldehyde/1% glutaraldehyde/PBS (in order to preserve fine actin structures such as filopodia), permeabilized in 0.2% Triton X-100/PBS, blocked with sodium borohydride (0.5 mg/ml) in PBS, and stained with rhodamine-conjugated phalloidin (0.1 μg/ml) in PBS. Cells for immunostaining were fixed with 4% paraformaldehyde/PBS, permeabilized, blocked with sodium borohydride, and incubated with primary antibodies diluted in PBS for 1 h at room temperature. Cells were immunostained to reveal vinculin, phosphotyrosine, and myc-tag as described previously (Nobes and Hall, 1995; Lamarche et al., 1996). To reveal c-fos, we used a rabbit polyclonal fos antibody (Oncogene Science Inc.) diluted 1:100 followed by FITC-conjugated goat anti–rabbit (1:200; Pierce and Warriner) and a tertiary layer of FITC-conjugated donkey anti–goat (1:200; Jackson ImmunoResearch Labs, Inc.). Dually phosphorylated ERK-1 and -2 were detected using monoclonal anti–MAP kinase, activated (Sigma Chemical Co.; M8159) and diluted 1:50, followed by FITC-conjugated goat anti–mouse (1:200; Pierce and Warriner) and a tertiary layer of FITC-conjugated donkey anti–goat. Cells were stained for myosin II using affinity-purified rabbit anti–human platelet nonmuscle myosin II antibody (Biomedical Technologies, Inc.) diluted 1:100. To detect expressed Ras protein, cells were permeabilized using 0.1% saponin/80 mM potassium Pipes (pH 6.8)/1 mM MgCl$_2$/5 mM EGTA for 2 min before fixation with 4% paraformaldehyde and incubation with rat monoclonal antibodies, Y13-238 (a kind gift of Mike Olson, Chester Beatty Laboratories), followed by Cy3-conjugated donkey anti–rat antibodies (Jackson ImmunoResearch Labs, Inc.). Injected cells were detected with Cascade blue avidin to biotin dextran which was coinjected with the Ras expression vector. Coverslips were mounted by inverting them onto 5 μl movial mountant containing *p*-phenylenediamine (a few grains/5 ml) as an antifade

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1. *Abbreviations used in this paper:* GAP, GTPase-activating protein; REF, rat embryo fibroblast.

agent. The coverslips were examined on a Zeiss axiophot microscope using Zeiss 10× and 20× air lenses, and 40× and 63 × 1.4 oil immersion lenses. Fluorescence images were recorded on Kodak T-MAX 400 ASA film.

Wound Closure Measurements

Semiquantitative measurements were made of control wounds ($t = 0$) and of control and inhibitor-treated wounds (after the control wound had closed). Three randomly chosen regions of a single wound (each 700 μm long), stained with rhodamine-conjugated phalloidin, were photographed at a magnification of 20. A mean wound width in micrometers was determined (by averaging the width every 30 μm) and an average percent wound closure was calculated.

For microinjection experiments, a region of wound was randomly chosen and a continuous stretch of 10–15 leading edge cells on opposing sides of the wound was microinjected. Since not only front row cells participate in the wound response, we also injected cells in rows 2, 3, and 4 on both sides of the wound. For microinjected wounds, wound width was measured as the distance between microinjected cells (on either side of the wound) visualized by coinjected fluorescent dextran or by anti-myc staining, since some noninjected cells often crawl between injected cells into the wound. In this case, measurements were made every 15 μm over a length of wound of ~300–400 μm. From the average values for each injection, percent wound closure was calculated.

Golgi Apparatus Reorientation Measurements

To record the position of the Golgi apparatus in migrating wound edge cells, wider wounds (~200 μm wide) were made to monolayers of REFs and cells were fixed 2, 4, and 6 h after wounding. The Golgi apparatus was localized by immunolabeling, as described above, using a rat monoclonal antibody (23C) which recognizes β-COP. The position of the immunolabeled Golgi apparatus in each cell was recorded as described in Kupfer et al. (1982). Wound edge cells were divided up into three 120° sectors centering on the nucleus on the wound (see Fig. 5 A). Cells in which the Golgi apparatus (i.e., 50% or more of the fluorescent image) was within the sector facing the wound were scored positive and for each time point at least 100 cells were examined. For the zero time point the random value of 33.3% of cells showing Golgi apparatus in the forward facing sector was given. To examine the effect of mutant GTPases on Golgi apparatus reorientation, wounds were injected with expression vectors encoding myc-tagged N17Rac, N17Cdc42, WASp, and V12Cdc42 1 h after the wound was made. Expressed myc-tagged proteins were detected 1 h later when 50% of cells repositioned their Golgi apparatus to the forward facing sector. The cells were fixed 5 h later and Golgi apparatus position was recorded in wound edge cells expressing myc-tag.

Time-Lapse Imaging of Wound Edge Cells

A CCD camera and time-lapse controller (EOS Electronics) were attached to a Zeiss inverted microscope. Injected or inhibitor-treated cells were placed in a slide flask (Cat. no. 170920; Nunc). Microscope images were collected at a rate of 10 frames every 60 s on a Sony betacam video recorder. Individual frames were transferred from videotape to a Macintosh computer with a frame grabber and processed using Adobe Photoshop. After time-lapse, cells were fixed and stained to identify microinjected cells.

Results

Analysis of Cell Movement in a Wounded Fibroblast Monolayer

To investigate the role of small GTPases in fibroblast motility, small scrape wounds were made across a confluent monolayer of primary REFs (Fig. 1 A). The wound width is approximately two to three cells across (mean 120 μm) and during a period of 5–6 h, cells move bodily forwards to fill the gap without the necessity for cell division (Fig. 1, B and D). The response to wounding is rapid; within 30 min, rounded and retracted cells at the wound edge respread
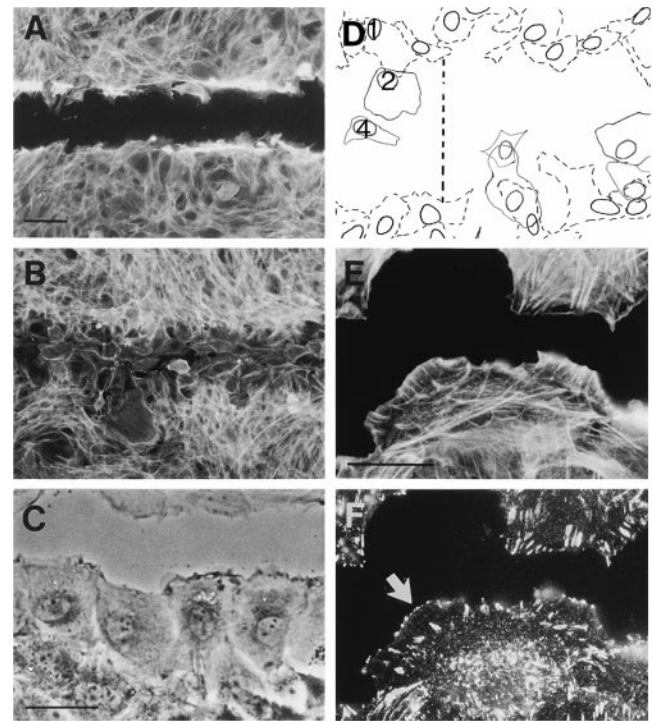

Figure 1. REF cell behavior at the wound edge. REF monolayers were fixed 5 min (A) and 5 h (B) after wounding and visualized with rhodamine-conjugated phalloidin. Bar, 120 μm. (C) Phase-contrast image of wound edge 3 h after wounding showing polarized morphology of front row cells. Bar, 20 μm. (D) Outline of three cells drawn from time-lapse recording after 15 min (dashed), 2 h, and 4 h as they migrate into the wound space (see numbers 1, 2, and 4 within nucleus of cell on left side). Dashed line across wound space is 125 μm. (E and F) High-power image of wound edge cell fixed 3 h after wounding and costained for actin filaments (E) and for phosphotyrosine (F). Wound edge cells display actin stress fibers, lamellipodia, filopodia (generally within lamellipodia), elongated focal adhesions, and focal complexes (arrow in F). Bar, 25 μm.

and extend filopodia and lamellipodia into the open space. 2 h after wounding, front row cells have clearly developed a polarized morphology, with lamellipodia and membrane ruffling localized only at the leading edge and not at the sides or rear edge of cells, where they are in contact with neighboring cells (Fig. 1 C). The wound edge cells remain fairly tightly packed (Fig. 1 C) and adherens junctions, visualized with cadherin antibodies, remain intact during the course of the assay (data not shown). Rhodamine-conjugated phalloidin reveals that all cells in the monolayer contain abundant stress fibers, but in addition, cells at the leading edge also have actin-rich lamellipodial and filopodial extensions (Fig. 1 E). Antiphosphotyrosine antibodies reveal elongated focal adhesions in all cells and cells at the leading edge also have smaller focal complexes intimately associated with lamellipodia and filopodia (see arrow in Fig. 1 F). After ~5 h, as opposing cells approach each other, time-lapse cinematography reveals that cell ruffling ceases within minutes of cell–cell contact (data not shown).

Wounding results in the rapid induction of various transcription factors such as c-fos and Egr-1 in cells both at the wound edge and several rows back into the monolayer (Verrier et al., 1986; Martin and Nobes, 1992; Pawar et al., 1995). We observe c-fos upregulation in four or five rows of cells back from the leading edge (data not shown). However, inhibition of protein synthesis, by pretreatment of the cells with cycloheximide (100 μg/ml), has no effect on wound closure (percent wound closure is 90 ± 3.6; control = 96 ± 2.9%) and we conclude that changes in gene transcription and protein synthesis are not required for forward movement in this short-term assay.

Rac Activity Is Essential for Protrusive Activity and Movement

Cell motility is generally associated with the protrusion of two types of actin-rich structure, lamellipodia and filopodia, at the leading edge of cells and in Swiss 3T3 fibroblasts, these are controlled by Rac and Cdc42, respectively (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). To determine whether Rac plays a role in wound-induced cell motility, patches of cells on opposite sides of a wound were microinjected with either dominant negative Rac (N17Rac) protein, or with an expression vector encoding N17Rac. Rhodamine-conjugated phalloidin staining revealed that inhibition of Rac blocked all lamellipodial activity (Fig. 2, compare B with A). Cells were left with a "scalloped" shape, although filopodia could still be seen (Fig. 2 B, arrow). Inhibition of Rac had severe effects
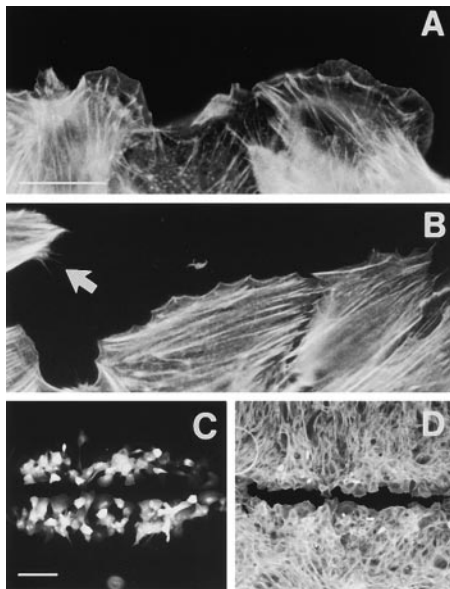
on cell movement (Fig. 2, C and D) and quantitative analysis (Fig. 3) revealed that in cells injected with N17Rac protein wound closure was blocked by ~80%, whereas in cells injected with the N17Rac expression construct cell movement was inhibited by 98%. The difference in these values likely reflects the instability of N17Rac protein (half-life ~3 h; Ridley et al., 1992). Microinjection of constitutively active Rac protein (V12Rac) had no inhibitory effect on cell movement (Fig. 3). We conclude that Rac is activated upon wounding and that Rac activity is essential for cell movement.

Cdc42 Activity Is Required to Establish Polarity during Movement

To determine whether Cdc42 also plays a role in wound closure, patches of cells on opposite sides of a wound were injected with either dominant negative Cdc42 (N17Cdc42) protein, or with an expression vector encoding N17Cdc42. It can be seen from Fig. 3 that inhibition of Cdc42 results in a 50% inhibition of cell movement. Microinjection of cells with the Cdc42-binding domain of the Cdc42 target protein, WASp, resulted in a similar level of inhibition (Fig. 3), confirming that Cdc42 is required for efficient movement but is not absolutely essential.

To examine the role played by Cdc42 during wound closure, we looked more carefully at migrating cells lacking Cdc42 activity. Fig. 4 A shows the typical polarized morphology of a wound edge cell (visualized with injected fluorescent dextran) with ruffling activity restricted to the front edge. In contrast, Fig. 4 B shows the morphology of a wound edge cell in which Cdc42 has been inhibited. Cell polarity is completely lost and protrusive, lamellipodial ac-
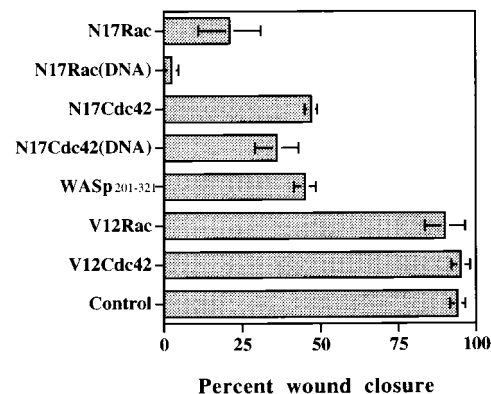

Figure 2. Rac is required for lamellipodial protrusions. Control wounds (A) and N17Rac (1 mg/ml)-injected wounds (B–D) were fixed and actin filaments visualized with rhodamine-conjugated phalloidin. Arrow in B indicates filopodia extending from an N17Rac-injected cell. Bar in A, 20 μm. Wound assay from which measurements of wound closure are made is shown in C and D. 1 h after wounding, wound edge cells were coinjected with N17Rac and injection marker (fluorescent dextran, C). Wounds were fixed ~5–6 h later when the control injected wounds had closed. Bar in C, 200 μm.

Figure 3. Effects of activating or inhibiting Rac and Cdc42 on wound closure. REF monolayers were wounded and the activity of Rac and Cdc42 in wound edge cells was modulated by microinjection of N17Rac (1 mg/ml), N17Cdc42 (2 mg/ml), WASp fragment (2 mg/ml), V12Rac (1 mg/ml), V12Cdc42 (1 mg/ml) proteins, or expression vectors encoding the same (indicated by DNA in parentheses). Percent wound closure was calculated as described in Materials and Methods. Experimental wounds were fixed at the same time that control wounds closed and stained with rhodamine-conjugated phalloidin to visualize actin filaments and wound space. Cells were coinjected with fluorescent dextran in order to visualize the position of injected wound edge cells. Values represent means ± SEM for at least three independent experiments.
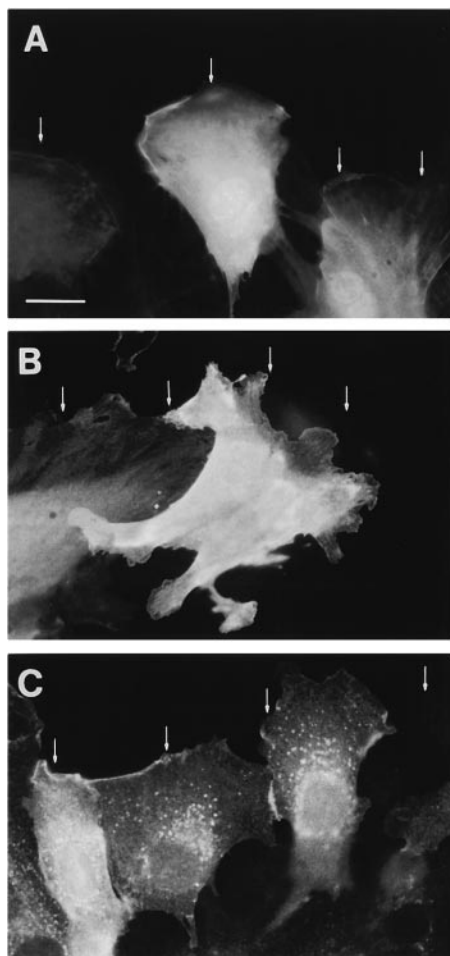
Figure 4. Cdc42 is required for morphological polarization in leading edge cells. 1 h after wounding, wound edge REFs were microinjected with fluorescent dextran (2 mg/ml) (A) or a myc-tagged expression vector encoding WASp fragment to inhibit Cdc42 activity (B) or a myc-tagged expression vector encoding V12Cdc42 (C). Cells were fixed 3 h later when wounds were still open and myc-tagged proteins were visualized by indirect immunofluorescence. The position of the wound edge in A–C is indicated by arrows. Bar, 20 μm.
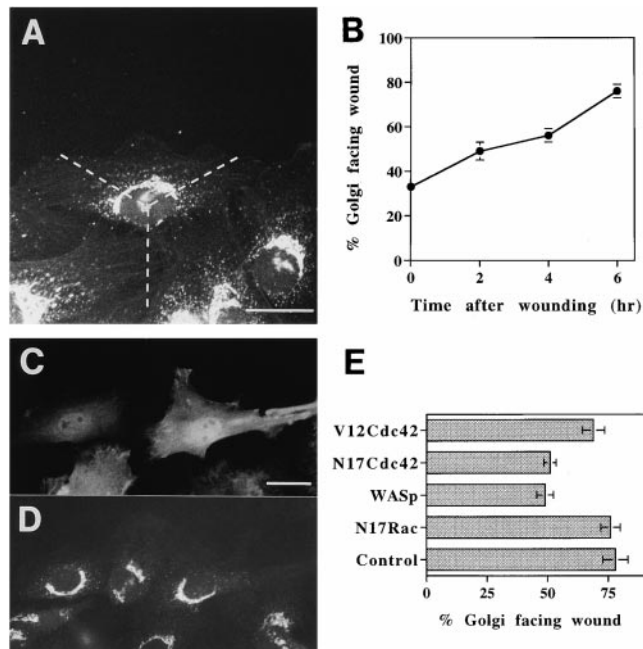

Figure 5. Cdc42 is required for reorientation of the Golgi apparatus. (A) REFs fixed and stained using indirect immunofluorescence to show the distribution of the Golgi apparatus 4 h after wounding. The percentage of cells having their Golgi apparatus in the forward-facing 120° sector was measured 2, 4, and 6 h after wounding (B). Random orientation of the Golgi apparatus with respect to the wound edge corresponds to 33% at 0 h (B). Wound edge cells were microinjected with expression vectors encoding myc-tagged V12Cdc42, N17Cdc42, WASp fragment, or N17Rac 1 h after wounding. Expression of myc-tagged proteins could first be detected 1 h later, at which time 49% of first row control wound edge cells had their Golgi apparatus facing the wound. Cells were fixed 6 h after wounding and double-labeled to reveal expression of myc-tagged proteins and the Golgi apparatus (shown for myc-tagged WASp fragment in C and corresponding Golgi apparatus in D). Percentage of cells with Golgi apparatus facing the wound was calculated for each condition (E). For each time point and/or experimental condition, at least 100 cells from three different experiments were scored. Values represent means ± SEM. Bar, 20 μm.

tivity can be seen all around the cell's periphery. Expression of constitutively active Cdc42 (V12Cdc42) protein had no effect on cell polarity (Fig. 4 C) or forward movement (Fig. 3).

The polarized morphology of migrating cells also involves the reorganization of the microtubular network and the alignment of the Golgi apparatus in the direction of movement (Gotlieb et al., 1981, 1983; Kupfer et al., 1982; Euteneuer and Schliwa, 1992; Bershadsky and Futerman, 1994). Although no major reorganization of the microtubular network could be seen during the REF wound healing assay (data not shown), disruption of microtubules with nocodazole did inhibit wound closure by 60 ± 6% (n = 3). Realignment of the Golgi apparatus, on the other hand, could be clearly observed during the assay. As seen in Fig. 5, A and B, during the course of the experiment the percentage of cells with their Golgi apparatus orientated in the direction of movement rose from 33% (essentially ran-

dom) to >80%. Although the relatively long time course suggests that this realignment is not essential for movement, it may facilitate movement and is a clear read-out of a developing polarized morphology. To determine whether Cdc42 activity is required for this aspect of cell polarity, Golgi apparatus reorientation was measured in wound edge cells after injection with dominant negative Cdc42 or with the WASp fragment. Fig. 5 (C–E) shows that inhibition of Cdc42 completely prevents Golgi apparatus realignment. Wound-induced Golgi apparatus reorientation was not inhibited by constitutively active Cdc42 (V12Cdc42) or by dominant negative Rac (N17Rac) (Fig. 5 E).

Rho and Cell Movement

In Swiss 3T3 fibroblasts, Rho is required for both the formation and maintenance of actin stress fibers and focal adhesions (Ridley and Hall, 1992; Hotchin and Hall, 1995).
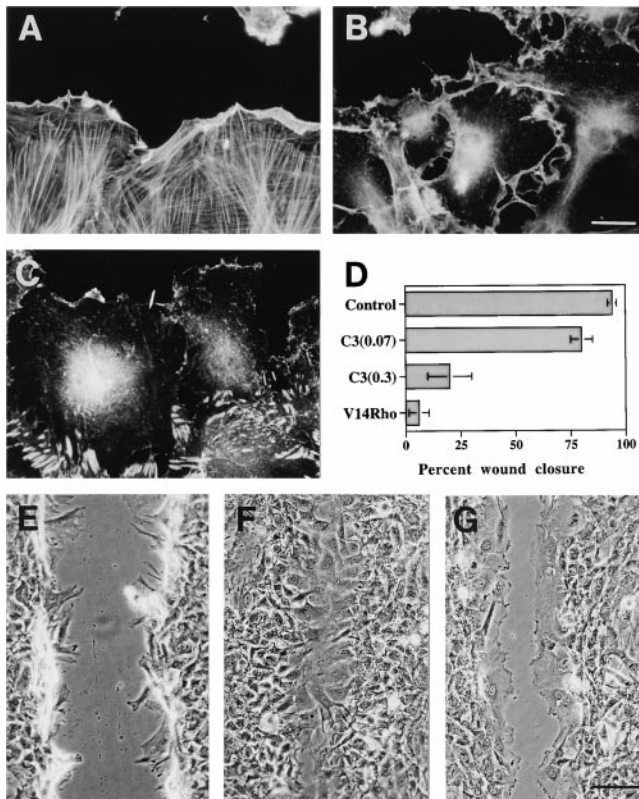
Figure 6. Rho is required for cell substrate adhesion but focal adhesions and stress fibers inhibit movement. Wounded REFs untreated (A) or injected with C3 transferase (0.07 mg/ml) 1 h after wounding (B and C) were fixed 2 h later and actin filaments were visualized with rhodamine-conjugated phalloidin (A and B) and vinculin was visualized by indirect immunofluorescence (C). (D) Percent wound closure was calculated for wounds injected with dextran (Control), C3 transferase (0.07 and 0.3 mg/ml), and V14Rho protein (0.4 mg/ml). Values represent means ± SEM for at least three different experiments. Bar, 20 μm. Phase-contrast images of wounded REFs 15 min after wounding (E) and 4 h after wounding in the presence (F) or absence (G) of Y-27632 (20 μm). Bar, 100 μm.

REF monolayers have abundant stress fibers, and after wounding motile edge cells continue to display many actin stress fibers oriented both towards the wound and radially across the cell (Fig. 6 A). To determine whether these structures are required for cell movement, cells were microinjected with the Rho inhibitor, C3 transferase, at a concentration of 65–75 μg/ml. As shown in Fig. 6, B and C, at this concentration of C3 transferase, cells are devoid of all visible stress fibers and focal adhesions, yet they continue to extend lamellipodia and filopodia normally and wounds close (Fig. 6 D).

Although Rho activity, as visualized by stress fiber formation, is not required for wound closure, microinjection of C3 transferase at a fourfold higher concentration (300 μg/ml) results in loss of substrate adhesion and severe cell retraction (data not shown) and under these conditions wound closure is significantly inhibited (80% inhibition, see Fig. 6 D). Using this experimental approach, we were unable, therefore, to assess whether the absence of actin

stress fibers/focal adhesions could enhance cell movement, since inhibition of Rho with C3 transferase has at least two distinct effects. However, stress fibers and focal adhesions can be inhibited more directly by blocking the activity of one of the downstream targets of Rho, p160ROCK (Uehata et al., 1997), with compound Y-27632. In the presence of 20 μM Y-27632, wound edge cells contained few actin stress fibers (data not shown), but remained attached to the coverslip (Fig. 6 F). Under these assay conditions, there is a significant increase in the speed of wound closure by ~30% as indicated by the position of the wound edges in the presence (Fig. 6 F) and absence (Fig. 6 G) of Y-27632.

Finally, microinjection of a constitutively active Rho protein (V14RhoA), which is unable to cycle through an inactive GDP-bound state, severely inhibits wound closure (95% inhibition; see Fig. 6 D). Injection of V14Rho does not obviously increase the number of actin stress fibers in wound edge cells, which are already abundant, although in some cases stress fibers and focal adhesions appear to be thicker than in control cells (data not shown).

We conclude that Rho-dependent stress fibers and focal adhesions are not required for cell movement, but that a basal Rho activity is required to maintain cell substrate adhesion as previously described (Takaishi et al., 1993; Ridley et al., 1995; Santos et al., 1997). Furthermore, focal adhesions and stress fibers must undergo dynamic turnover during movement and this requirement results in an inhibitory effect on the speed of movement.

Ras Activity Is Required for Cell Movement

Ras has been proposed to play a role in regulating the turnover of focal adhesions (Schlaepfer and Hunter, 1998). To determine whether Ras is activated during the assay, wounded cells were visualized with an antibody that recognizes dually phosphorylated ERK, a downstream target of the Ras pathway. Fig. 7 shows that ERK is activated rapidly after wounding (within 5 min), but that the levels decrease to background by 1 h. Interestingly, ERK activation is seen to extend further back from the wound edge than c-fos induction (eight rows as opposed to only four). Wound-induced ERK activation can be blocked by microinjection of the neutralizing Ras antibody, Y13-259 (data not shown), or by preincubation with the MEK inhibitor (PD98059) (Fig. 7), indicating a requirement of the Ras/MEK pathway for wound-induced ERK activation.

To determine whether Ras is required for cell movement, patches of cells around a wound were microinjected with the neutralizing Ras antibody Y13-259 (Furth et al., 1982). Inhibition of Ras has no effect on lamellipodia protrusions (Fig. 8, A and B), nor does it affect cell polarity (data not shown). Nevertheless, cell movement is inhibited by 80% (Fig. 8 G). Further analysis by time-lapse videomicroscopy of cells in which Ras has been inhibited revealed normal protrusive activity, but no net cell movement. However, we did observe that actin stress fibers and focal adhesions in the Ras-inhibited cells (Fig. 8, A–D) appeared to be somewhat thicker and larger than those in control cells, similar to what we observed in cells injected with constitutively active Rho. These observations suggested a possible link between Ras and focal adhesion and
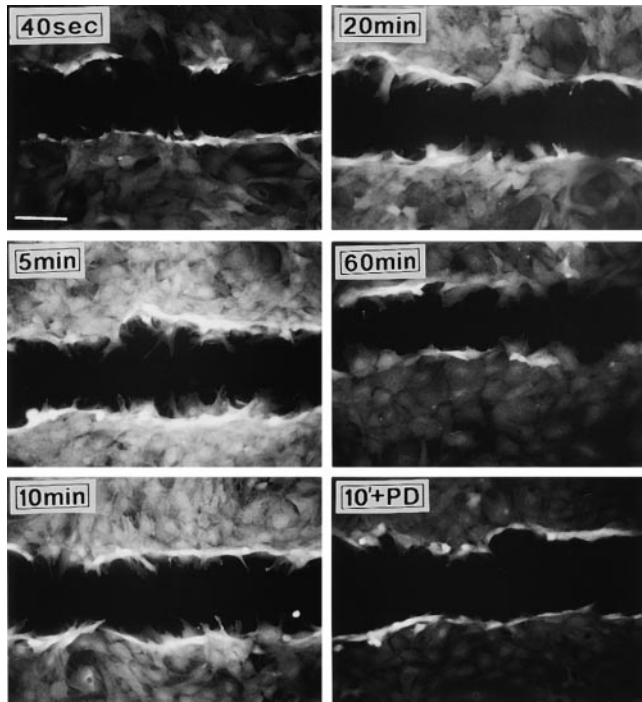
Figure 7. Ras and ERK are activated after wounding. REF wounds were fixed 40 s, 5, 10, 20, and 60 min after wounding. Dually phosphorylated ERK was visualized by indirect immunofluorescence. Cells were pretreated with the MEK inhibitor PD98059 (60 μM) before wounding and then fixed 10 min after wounding and stained to reveal phosphoERK (10′+PD). Bar, 100 μm.

Figure 8. Ras is required for cell movement. 1 h after wounding, wound edge cells were coinjected with the neutralizing Ras antibody Y13-259 (9 mg/ml) and fluorescent dextran as an injection marker (A–D). Cells were fixed 3–4 h later and actin filaments were visualized with rhodamine-conjugated phalloidin (A) and vinculin was visualized by indirect immunofluorescence (C). Arrows in C indicate larger focal adhesions in the Ras antibody–injected cell. Injection marker is shown in B and D. Expressed Ras was localized using indirect immunofluorescence (E) and cells were costained to reveal vinculin (F). Percent wound closure was calculated for wounds injected with V14Rho (0.4 mg/ml), anti-Ras antibody (8 mg/ml), anti-Ras antibody (8 mg/ml) + C3 transferase (0.06 mg/ml), anti-Ras antibody (8 mg/ml) in the presence of Y-27632 (20 μm) (Anti-Ras+Y), and dextran (Control) (G). Wounds were pretreated with inhibitors PD98059 (60 μM) and LY294002 (20 μM) for 20 min before wounding. Percent wound closure for these inhibitors compared with control wounds is shown in H. Bar, 20 μm.

stress fiber turnover. To test this, cells were injected with the Ras inhibitor Y13-259 and were either coinjected with the Rho inhibitor C3 transferase, or were treated with the p160ROCK inhibitor Y-27632. Under both conditions, stress fibers and focal adhesions were lost as expected (data not shown), but now cells migrated normally resulting in wound closure (Fig. 8 G).

Interestingly, when V12Ras is expressed at relatively low levels and at early times (2 h) after microinjection of an expression construct, we clearly observe Ras concentrated at focal adhesion sites using the anti-Ras antibody, Y13-238 (see Fig. 8 E and vinculin costain in Fig. 8 F). After longer times (6 h), V12Ras has a significant affect on cell morphology, inducing loss of adhesions and changes in cell shape, typical of a transformed morphology.

Ras regulates a number of signaling cascades including the ERK-MAP kinase pathway and in some, but not all, cells PI3-kinase (Rodriguez-Viciana et al., 1997). To examine whether either of these two activities might be responsible for the Ras requirement seen here, we have used the MEK inhibitor PD98059 and the PI3-kinase inhibitor LY294002. Either of these inhibitors alone results in a relatively weak inhibition of wound closure (25 and 30%, respectively; see Fig. 8 H) and even in combination, wound closure is only inhibited by ~50%. We conclude that neither of these downstream pathways is likely to account for the specific Ras requirement during wound closure.
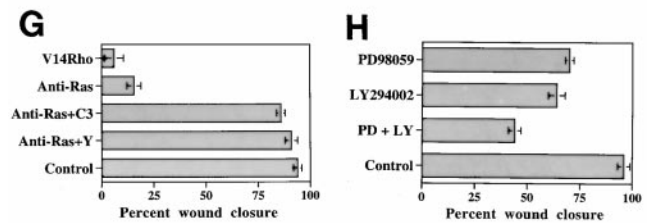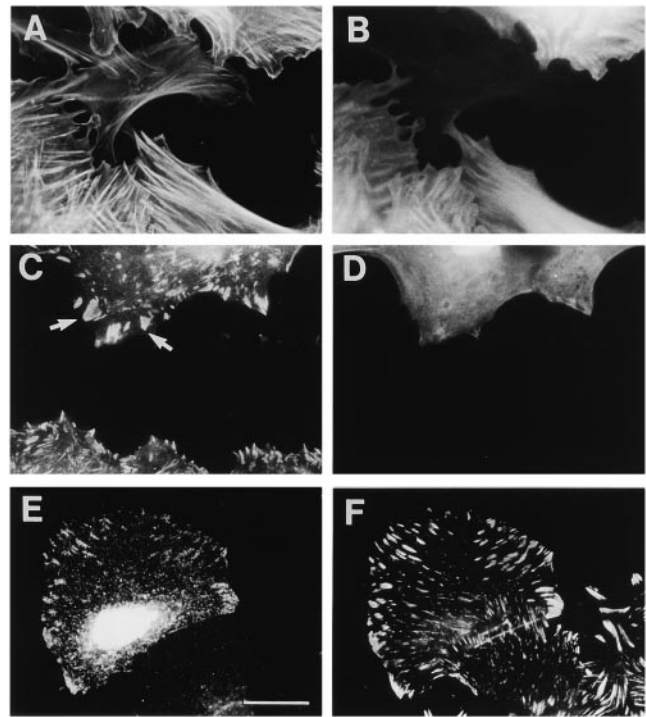
Discussion

The analysis of a model cell line, Swiss 3T3 fibroblasts, has led to the identification of three signal transduction pathways responsible for controlling the organization of the actin cytoskeleton and each is regulated by a member of the Rho family of small GTPases (reviewed in Ridley, 1996; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Rho promotes the assembly of actin–myosin contractile filaments (stress fibers) and associated focal adhesion com-

plexes, Rac induces the formation of protrusive lamellipodial structures (Ridley and Hall, 1992; Ridley et al., 1992), while Cdc42 triggers filopodia formation (Kozma et al., 1995; Nobes and Hall, 1995). Since the assembly and disassembly of filamentous actin structures drive cell movement, axon guidance, phagocytosis, and cytokinesis, it is likely that Rho GTPases will play key roles in controlling these fundamental biological processes. Here we examine the individual contributions of Rho, Rac, and Cdc42 to cell movement.

Animal cells move by crawling along a surface and this involves a combination of protrusive activity at a leading edge combined with forward movement of the nucleus and cell body (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Cells may move either as individuals or as a group, often in sheets, and examples of both types of movement can be found in the embryo and in the adult. To analyze how cells move cooperatively, we have used an in vitro wound healing assay. Individual cells in a confluent fibroblast monolayer are nonmotile and appear to have low levels of Rac and Cdc42 activity since only a few, sporadic membrane protrusions can be seen. Rho, on the other hand, appears to be highly active, since cells display many stress fibers and focal adhesions. Wounding of the monolayer induces cells at or close to the leading edge to crawl forward to close the gap and during the 5-h course of our experiments, the cells move cooperatively as a sheet, retaining close contacts with their neighbors.

The most obvious and immediate effect of wounding is to induce (within minutes) dynamic activity, lamellipodial and filopodial protrusions, and membrane ruffling, at a leading edge. This is consistent with a wound-induced activation of Rac and Cdc42, although it is formally possible that these GTPases are already active in the monolayer, but functionally silent due to cell–cell contacts. In any event, microinjection of wound edge cells with a Rac inhibitor prevents lamellipodia formation and membrane ruffling (filopodia are still seen) and there is no forward movement over the time course of the experiment. Others have reported that Rac is required for chemotaxis of fibroblasts towards PDGF (Anand-Apte et al., 1997) and macrophages towards CSF-1 (Allen et al., 1998), for hepatocyte growth factor–induced scattering of MDCK epithelial cells (Takaishi et al., 1994; Ridley et al., 1995), and for the invasive movement of some metastatic cells (Habets et al., 1994; Michiels et al., 1995). Furthermore, genetic disruption of Rac in the *Drosophila* embryo prevents the actin cytoskeleton changes required to drive dorsal closure, a process involving the movement of sheets of epithelial cells around the early embryo (Riesgo-Escovar et al., 1996; Glise and Noselli, 1997). It appears, therefore, that Rac may be universally required to drive cell crawling, consistent with its unique role in controlling the formation of lamellipodia.

The other protrusive structures seen after wounding are filopodia and, as expected from work with Swiss 3T3 cells, these can be completely blocked by inhibition of Cdc42 in wound edge cells. However, this results in only a partial block (~50%) of wound closure and it appears that although Cdc42 activity is required for efficient cell movement, in this assay it is not absolutely essential. Nevertheless, closer inspection of cells in which Cdc42 has been

inhibited reveals an extremely interesting role for this protein. Normally, cells at the wound margin develop a morphological polarity showing a clear leading edge (with membrane ruffles and filopodia), but no protrusional activity at their sides or rear, where they are in contact with neighboring cells. In addition, migrating cells polarize their Golgi apparatus to a position forward of the cell nucleus and in the direction of migration (see also Kupfer et al., 1982) and disruption of the Golgi apparatus directly with brefeldin A, or indirectly with nocodazole (to disrupt microtubules) inhibits directed cell migration (Bershadsky and Futerman, 1994). We observed that inhibition of Cdc42 in wound edge cells caused a complete loss of polarity; cells protrude lamellipodia all around their periphery, irrespective of whether there are cell–cell contacts, and Golgi apparatus reorientation does not occur. We conclude that Cdc42 is not required to activate Rac, but that it is required to restrict Rac activity to the leading edge through the generation of a polarizing signal.

These observations point to some interesting analogies with the yeast *Saccharomyces cerevisiae*, where Cdc42 is required for the formation of the bud during cell growth (Drubin, 1991; Drubin and Nelson, 1996). Inactivation of Cdc42 causes a disruption of the actin cytoskeleton and a loss of cell polarity and directed secretion; as a consequence, cell growth becomes isotropic (Adams et al., 1990). These results bare striking similarities with what we observe in the wound assay. However, one significant difference is the effect of a constitutively active Cdc42; in yeast this also causes loss of cell polarity (Ziman et al., 1991) but in wound edge cells it has no obvious effect on their polarity. Perhaps the presence of cell–cell contacts in fibroblast monolayers provides an additional constraint, and in this respect, it is interesting to note that both dominant negative and constitutively activated Cdc42 prevent directed cell migration, though not motility per se, of isolated macrophages in a chemotaxis assay and reorientation of the MTOC in isolated T cells after interaction with antigen-presenting cells (Stowers et al., 1995; Allen et al., 1998). Our observations point to some interesting interplay between Cdc42 activity and cell–cell contacts.

The mechanism by which Cdc42 generates polarity in multicellular organisms is unknown, although in yeast, Cdc42 is directed to the bud site by Bud1, another small GTPase in the Ras family. It is not even clear whether filopodia are required or whether they are formed after polarity has been established. Our experiments suggest two extreme roles for Cdc42: (a) it directs protrusion (i.e., Rac activity) and secretion (microtubule-dependent Golgi apparatus reorientation) to the leading edge; or (b) it is required for inhibition of protrusive activity from regions involved in cell–cell contact. By analogy with yeast, we favor the first model but we cannot exclude the second.

Contractile actin–myosin filaments are found in all animal cells; in cultured fibroblasts these are readily seen as well organized bundles (stress fibers), tethered to the plasma membrane at specialized sites (focal adhesions) (Jockusch et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Burridge et al., 1997). The REFs used in the wound healing assay have abundant stress fibers and focal adhesions and these are maintained even in leading edge cells after wounding. It is often said that these structures

likely inhibit locomotion (Couchman and Rees, 1979), and we have confirmed this using the Y-27632 compound, an inhibitor of the Rho-effector p160ROCK which mediates actin stress fiber assembly (Leung et al., 1996; Amano et al., 1997; Ishizaki et al., 1997; Uehata et al., 1997). Y-27632 induces a loss of actin stress fibers and focal adhesions (without obviously affecting cell shape or attachment) and causes a significant (30%) increase in the rate of cell migration. Interestingly, the myosin ATPase inhibitor BDM, which also stimulates loss of actin stress fibers, inhibited cell motility by 30% (data not shown), suggesting that other contractile, actin–myosin filaments may play some role during cell movement in this assay.

Several groups have identified an essential role for Ras in cell movement, both in chemotaxis and in wound closure assays (Sosnowski et al., 1993; Fox et al., 1994; Kundra et al., 1995). We also find that inhibition of Ras blocks cell movement, but we also show that movement can be fully restored by simultaneous inhibition of focal adhesion/stress fiber assembly by inhibiting either Rho or its effector protein, p160ROCK. This experiment strongly suggests that Ras regulates the turnover of focal adhesions and stress fibers, clearly an important feature of migrating cells (Izzard and Lochner, 1976; Dunlevy and Couchman, 1993). This could occur in at least two ways. First, Ras might regulate the Rho GTPase cycle. Since Rho is required for the formation of focal adhesions, their turnover may depend on Rho inactivation (i.e., formation of Rho.GDP from Rho.GTP), which is likely to be controlled by a GTPase-activating protein (GAP). One obvious candidate molecule that could provide a link between the two GTPases is p120RasGAP, which is known to exist in a complex with p190RhoGAP, a GAP active on Rho. In fact, overexpression of the NH_2-terminal, non-GAP containing region of p120RasGAP leads to loss of stress fibers (Ellis et al., 1990; Settleman et al., 1992; McGlade et al., 1993; Ridley et al., 1993).

The second possibility is that Ras regulates focal adhesion turnover independently of Rho. In support of this model is the observation that although the p160ROCK inhibitor Y-27632 restores cell movement after Ras inhibition, it does not restore cell movement in cells expressing constitutively activated Rho protein (V14Rho) (data not shown). However, the mechanism by which Ras might regulate focal adhesion turnover is not clear. Inhibition of MEK or PI3-kinase (two downstream pathways controlled by Ras) appears to have only minor effects on movement. This is in contrast to previous reports suggesting that PI3-kinase and the MAP kinase pathway are important for motility (Keely et al., 1997; Klemke et al., 1997). Furthermore, the components of the focal adhesion complex that are the target of Ras-mediated signals are also not clear, although focal adhesion kinase is one molecule that has been suggested to play an important role in their turnover (Ilic et al., 1995; Gilmore and Romer, 1996).

In conclusion, we have shown that Rac is essential for producing the leading edge protrusions required for forward movement in a wound healing assay. Cdc42 activity is essential to maintain the polarized phenotype of migrating cells and Rho is required for cell adhesion. Ras is also essential for promoting focal adhesion turnover during migration. Major outstanding questions concern the nature of the biochemical pathways that mediate these GTPase functions and how they are coordinated in time and space.

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