A Rac switch regulates random versus directionally persistent cell migration

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Directional migration moves cells rapidly between points, whereas random migration allows cells to explore their local environments. We describe a Rac1 mechanism for determining whether cell patterns of migration are intrinsically random or directionally persistent. Rac activity promoted the formation of peripheral lamellae that mediated random migration. Decreasing Rac activity suppressed peripheral lamellae and switched the cell migration patterns of fibroblasts and epithelial cells from random to directionally persistent. In three-dimensional rather than traditional two-dimensional cell culture, cells had a lower level of Rac activity that was associated with rapid, directional migration. In contrast to the directed migration of chemotaxis, this intrinsic directional persistence of migration was not mediated by phosphatidylinositol 3′-kinase lipid signaling. Total Rac1 activity can therefore provide a regulatory switch between patterns of cell migration by a mechanism distinct from chemotaxis.

Introduction

Cell migration is essential for normal embryonic development, immune system function, and tissue repair, but it also contributes to inflammatory diseases and tumor cell invasion (Lauffenburger and Horwitz, 1996; Ridley et al., 2003; Raftopoulou and Hall, 2004). Both the speed and the directionality of cell motility regulate migration, which is a complex process that includes the formation of membrane protrusions termed lamellipodia and lamellae at the leading edge of the cell to mediate forward advancement of the cell, membrane adhesive interactions with the migratory substrate, and coordinated dynamics of the cytoskeleton (Lauffenburger and Horwitz, 1996; Sheetz et al., 1999; Pollard and Borisy, 2003; Ridley et al., 2003).

Directional migration (i.e., cell motility in one direction) can involve either externally directed migration during chemotaxis or the intrinsic propensity of cells to continue migrating in the same direction without turning (i.e., intrinsic persistence of migration). Directional migration appears to be regulated by multiple mechanisms, including microtubules (Vasiliev et al., 1970; Goldman, 1971; Dujardin et al., 2003), Cdc42 (Nobes and Hall, 1999; Etienne-Manneville and Hall, 2003), integrins (Danen et al., 2005), and chemotactic stimuli (Haugh et al., 2000; Franz et al., 2002; Weiner, 2002). Chemotaxis imposes faster "directed migration" on cells through local activation of Rac or Ras by an external chemical or protein signal, activation of phosphatidylinositol 3′-kinase (PI3K), and establishment of a phosphoinositide gradient (Srinivasan et al., 2003; Sasaki et al., 2004; Van Haastert and Devreotes, 2004).

However, many migratory processes in development and tissue remodeling occur with no evidence of extrinsic chemotactic signaling, but instead by using intrinsic cell migration properties (Trinkaus, 1969). Rac and Rho are well-known modulators of various types of cell migration including chemotaxis, but their role in regulating intrinsic persistence and directionality of migration is not clear (Evers et al., 2000; Chung et al., 2000; Etienne-Manneville and Hall, 2002; Fukata et al., 2003; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004; Weiss-Haljiti et al., 2004).

In this study, we examined the following fundamental question: Is there a basic, intrinsic cellular mechanism that regulates whether a cell will migrate relatively straight ahead or in randomly changing directions? That is, what intracellular

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Abbreviations used in this paper: 2D, two-dimensional; 3D, three-dimensional; PI3K, phosphatidylinositol 3′-kinase; si, small interfering.

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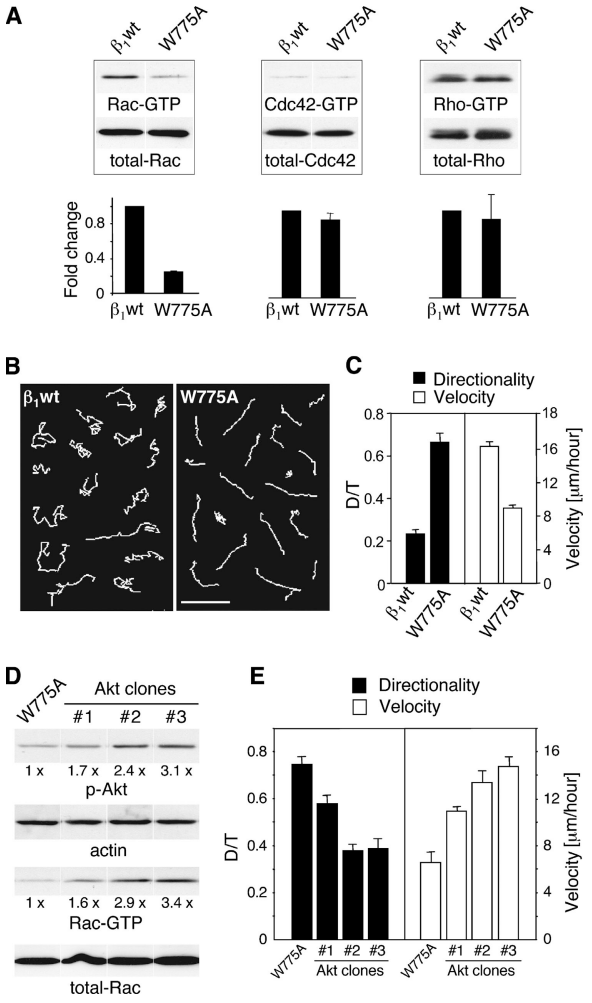

Figure 1. **Integrin mutation inhibits Rac signaling and suppresses random cell motility.** (A) GD25 cells with a point mutation in the β_1 integrin cytoplasmic domain (W775A) have a selective defect in Rac activation. Activities in pull-down assays for Rac-GTP, Cdc42-GTP, and Rho-GTP and total amounts of each protein in lysates of cells cultured on 1 μg/ml fibronectin were analyzed using antibodies against Rac, Cdc42, and Rho. Densitometry for each GTP-bound protein was normalized to the amount of the total protein, and results are presented as fold change compared with cells expressing wild-type integrin. The reduction of active Rac was 75% ($P < 0.0001$), whereas active Cdc42 and active Rho were not significantly reduced (by 10%, $P = 0.31$ and 8%, $P = 0.79$, respectively). In this and subsequent figures, all adjacent gel lanes are from the same gel and blot, but their order may be rearranged for clarity as indicated by white lines between the lanes. (B) Representative examples of migration tracks of cells expressing wild-type β_1 ($\beta_1$wt) or mutant (W775A) integrins cultured on fibronectin and tracked for 12 h. In these and subsequent composite migration figures, randomly selected individual migration tracks were copied and combined into a single figure to avoid empty spaces. Bar, 100 μm. (C) Quantification of the persistence of migratory directionality and velocity. Cell movements were recorded by time-lapse video microscopy and quantified by MetaMorph software. D/T ratios represent the ratio of the direct distance from start to end point [D] divided by the total track distance [T]. Motility was calculated as velocity (μm/h). Data were pooled from four independent experiments; error bars indicate SEM based on $n = 42–45$ cells. (D) Activated Akt can restore Rac activity. W775A cells were transfected with constitutively activated Akt, and stable clones were selected by serum starvation for 1 wk to induce apoptosis in cells with low Akt activity. The activity of Akt in the isolated clones (Akt clones 1–3) was assayed by immunoblotting with antibodies against phosphorylated Akt (p-Akt). Densitometry values for phospho-Akt were normalized to actin in the lysates, and the changes were calculated as fold increases compared with the parental W775A cells. The activity of Rac in the isolated Akt clones was calculated as the fold increase above W775A cell values after normalization of densitometry of the pull-down samples (Rac-GTP) to the total Rac in the lysates. (E) Activated Akt restores random motility. Quantification of cell movements of the Akt clones was performed as described in C. The decreases in D/T ratio after activated Akt expression were all significant (Akt clone 1, $P < 0.01$; clone 2, $P < 0.001$; and clone 3, $P < 0.001$), as were the decreases in velocity (clone 1, $P < 0.01$; and clones 2 and 3, $P < 0.001$). Error bars indicate SEM.

signaling mechanism determines whether a cell has an intrinsic pattern of directionally persistent migration or random, exploratory migration? We find that a relatively small change in total Rac activity can serve as a switch between these two patterns of cell migration in multiple cell types. Moreover, culturing fibroblasts in a three-dimensional (3D) compared with a two-dimensional (2D) environment was found to modulate Rac activity and change intrinsic directionality of cell migration.

Results

Integrin signaling, Rac, and migration

A potential mechanism for regulating intrinsic cell migration involving Rac was identified in our ongoing studies of an integrin point mutation that selectively affects an Akt–PKB pathway (Pankov et al., 2003). Fig. 1 shows that this specific integrin mutation also selectively suppresses downstream Rac activity, which is accompanied by a substantial suppression of random motility and enhanced persistence of migratory directionality. Specifically, Rac-GTP levels were decreased by 75% with no differences in total Rac protein (Fig. 1 A).

Loss of random motility and increased directionality of migration can be quantified by determining the ratio of the shortest, linear distance from the starting point of a time-lapse recording to the end point (D) compared with the total distance traversed by the cell (T). This D/T directionality ratio was increased by >2.8-fold in cells expressing the mutant integrin (Fig. 1, B and C; $P < 0.0001$), accompanied by a 45% decrease in the velocity of cell migration. The striking loss of random motility in the integrin mutant cells was confirmed using a mean square displacement assay (Gail and Boone, 1970; Fig. S1 A, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1). Notably, this loss of random cell motility associated with decreased Rac activity was not accompanied by any detectable change in either Cdc42 or Rho activity (Fig. 1 A).

Stable transfection with constitutively activated Akt compensated for the defect in Akt signaling and corrected most of the deficiency in active Rac (Fig. 1 D). Restoring levels of active Rac substantially decreased directionality and promoted random motility (Fig. 1 E). Transfection of mutant cells with low levels of constitutively activated Rac1 also restored random motility (Fig. S1, B and C), although higher levels proved inhibitory and cytotoxic (not depicted).

Small changes in Rac activity alter pattern of cell migration

To directly test the role of total Rac activation levels in regulating the directionality of cell migration in a range of different cell types, levels of Rac1 were knocked down using RNA

interference with small interfering (si) RNA. Reductions in Rac protein levels resulted in proportional changes in Rac activity (Fig. S2 [available at http://jcb.org/cgi/content/full/jcb.200503152.DC1] and not depicted). No effects of Rac1 knockdown on Cdc42 were detected, and Rho activity levels were generally unchanged (Fig. 2 A). Random cell migration was suppressed by such Rac1 knockdown in primary human fibroblasts, which is consistent with our findings in mouse ES cell–derived GD25 cells. The cells displayed increased directionality of migration, and suppression of overall velocity occurred only with a greater extent of Rac knockdown (Fig. 2, compare C with E; compare Video 1 with 2, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1). Similar results were obtained using either a pool of four Rac1 siRNA duplexes or individual Rac1 siRNA duplexes to reduce Rac1 activity (compare Fig. 2 with Fig. S3 A). Based on 11 independent experiments with primary human fibroblasts, a modest reduction in total Rac activity to 70% of original levels substantially enhanced directional persistence of migration, and 60% or lower levels produced maximal directionally persistent migration (Fig. 2 F). Conversely, overexpression of wild-type Rac1 or constitutively activated Rac1 in primary human fibroblasts promoted random cell migration with only minimal effects on overall velocity (Fig. S3, C and D). Using an independent approach to altering Rac activity, the Rac GEF inhibitor NSC 23766 reduced concentrations of active Rac in human fibroblasts and produced a loss of random motility with movements restricted to the long axis of the cells, followed by immobilization at high doses (unpublished data).

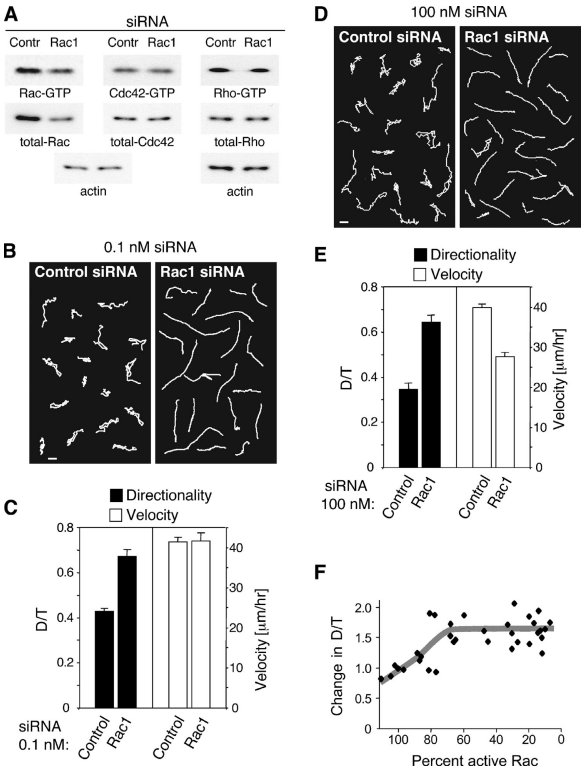

Figure 2. **Suppression of Rac1 expression lowers Rac activity and inhibits random motility of fibroblasts.** (A) Primary human fibroblasts were transfected with 1 nM Rac1 siRNA or 1 nM nonspecific control pool siRNA (Contr). Pull-down assays were performed as described for Fig. 1 after 72 h of siRNA treatment, using an immunoblot of lysates for actin to verify equal protein loading. The amount of active Rac (Rac-GTP) was reduced to 67% of original levels and the total amount of Rac protein (total-Rac) was reduced to 52% after this Rac1 siRNA transfection. In contrast, levels of active Cdc42 and Rho did not change. The immunoblot for total Rac was stripped and reprobed for total Cdc42 protein, which was not altered by the Rac1 siRNA. (B and D) Composite collection of representative migration tracks of human fibroblasts starting 72 h after transfection with control compared with either 0.1 or 100 nM Rac1 siRNA, respectively, tracked at 15-min intervals over a span of 10 h. Bars, 100 μm. (C and E) Quantification of persistence of migratory directionality (D/T) and velocity of the transfected cells at the two different siRNA concentrations: (C) 0.1 nM and (E) 100 nM. Error bars indicate SEM; the differences between control and 0.1 nM or 100 nM Rac1 siRNA D/T values were significant at the $P < 0.001$ level. (F) Increases in D/T ratios by reduction of active Rac using Rac siRNA were observed in all (11 out of 11) independent experiments, each testing three siRNA concentrations against the control. All data were pooled together for this composite graph based on video time-lapse microscopy on a total of 933 cells.

Regulation of directional persistence is specific to Rac

Cdc42 regulates filopodia formation (Nobes and Hall, 1995), and although it is involved in determining cell polarity (Nobes and Hall, 1999; Palazzo et al., 2001; Etienne-Manneville and Hall, 2003) and in regulating the directional migration of astrocytes in serum-free culture (Etienne-Manneville and Hall, 2001), knockdown experiments in human fibroblasts to selectively suppress Cdc42 levels and activity produced no loss of directional persistence of migration. Reducing the level of fibroblast Cdc42 protein and activity by >80% did not change Rac or Rho activity and also did not change either directionality or velocity of migration (Fig. 3, A and C, and not depicted). Rho stimulates cell contractility and adhesion by inducing the formation of actin stress fibers and focal adhesions, inhibition of Rho suppresses cell migration, and Rho can also affect directional persistence of migration in an epithelial cell system if cells express β_1 rather than β_3 integrins (Allen et al., 1998; Ridley et al., 2003; Danen et al., 2005). Although knocking down the RhoA level in human fibroblasts by >60% slowed migration speed by 40%, again there was no effect on Rac activity or on directionality, and the D/T ratio remained unchanged (Fig. 3, B and D). In addition, knockdown of Rac1 activity was not accompanied by any changes in the levels of β_1 or β_3 integrins (Fig. S4 A, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1), ruling out a mechanism involving integrin switching. Thus, only the overall levels of Rac activity could be linked specifically to the regulation of random versus directionally persistent migration under regular cell culture conditions.

Persistence of directional migration in epithelial and malignant cells

The mode of cell migration was found to depend on the relative level of Rac activity for two other types of cells (Fig. 4). After siRNA-induced reduction of Rac activity in the nontransformed human epithelial cell line MCF-10A, directional persistence was markedly increased (Videos 4 and 5, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1) with the D/T ratio increasing threefold (Fig. 4, A and B). In addition,

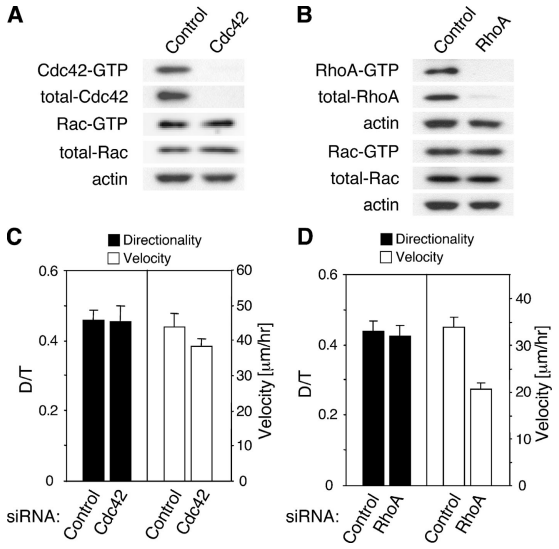

Figure 3. **Suppression of Cdc42 or RhoA expression does not affect directional persistence of cell migration.** (A and B) Specificity of Cdc42 and RhoA knockdowns. (A) 3 d after transfection of primary human fibroblasts with 100 nM Cdc42 siRNA, total Cdc42 protein levels were assayed by Western blotting with antibodies against Cdc42 in lysates of the transfected (Cdc42) and control cells treated with nonspecific siRNA (Control). Cdc42 siRNA led to a substantial decrease of both Cdc42 protein levels (total-Cdc42) and Cdc42 activity (Cdc42-GTP) by pull-down assay, whereas the activity and total amount of Rac in the same lysates (Rac-GTP and total-Rac) were not affected. (B) Primary human fibroblasts were transfected with 100 nM RhoA siRNA (RhoA) or nonspecific siRNA (Control). 3 d later, Rho protein levels were determined by Western blotting with antibodies against Rho. Although the levels of other Rho family members were not affected, the amount and activity of RhoA were substantially decreased. (C) After knockdown of Cdc42 by >80%, there were no significant changes in the D/T directionality ratio ($D/T_{Control} = 0.46 \pm 0.03$ and $D/T_{Cdc42} = 0.46 \pm 0.05$; $P_{D/T} = 0.89$) or the velocity ($V_{Contr} = 44.4 \pm 3.9$ μm/h and $V_{Cdc42} = 38.6 \pm 2.1$ μm/h; $P_V = 0.18$). (C and D) Error bars represent the SEM. (D) Suppression of RhoA expression affects the velocity of migration but not directionality. After reducing RhoA levels by at least 60% using siRNA, velocity of cell migration was reduced by 40% ($V_{Contr} = 34.4 \pm 2.4$ μm/h and $V_{RhoA} = 20.8 \pm 1.4$ μm/h; $P < 0.0001$), but directional persistence of migration was unaffected ($D/T_{Control} = 0.44 \pm 0.03$ and $D/T_{RhoA} = 0.42 \pm 0.03$; $P = 0.74$).

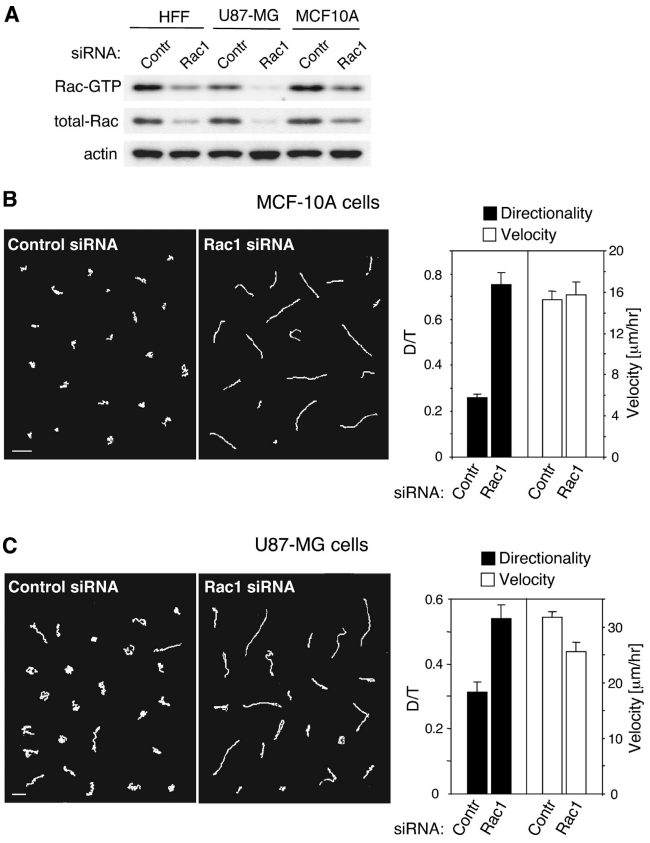

Figure 4. **Suppression of Rac1 results in increased directional persistence of migration in human epithelial and glioblastoma cells.** (A) Comparison of total and active Rac levels in three cell lines and effects of knockdown by Rac1 siRNA. Primary human foreskin fibroblasts (HFF), U87-MG human glioblastoma cells, and MCF-10A human mammary epithelial cells were transfected with 50 nM Rac1 pool siRNA or control siRNA, and then assayed for total and active Rac levels. (B) MCF-10A cells transfected with Rac1 siRNA migrate with much higher directional persistence with no change in velocity. MCF-10A cells were transfected with 50 nM Rac1 or control siRNA, detached with trypsin 72 h after transfection, replated at single-cell density, and recorded by time-lapse video microscopy. Suppression of Rac1 expression (Rac1 siRNA) induced straightening of cell migration tracks when compared with the cells transfected with the nonspecific siRNA (Control siRNA). Bar, 100 μm. The quantified directionality of Rac1 siRNA transfected cells (Rac1) increased threefold compared with cells transfected with control siRNA (Contr) ($D/T_{Control} = 0.26 \pm 0.02$ vs. $D/T_{Rac1} = 0.76 \pm 0.05$; $P < 0.0001$), whereas the velocities remained comparable ($V_{Contr} = 15.3 \pm 0.8$ μm/h vs. $V_{Rac1} = 15.7 \pm 1.3$ μm/h; $P = 0.79$). (C) U87-MG cells transfected with Rac1 siRNA lose random motility and migrate with greater directionality. U87-MG cells were transfected with 200 nM Rac1 siRNA, and after 3 d, cell migration was recorded. Bar, 100 μm. Rac1 suppression resulted in a significant increase in directionality ($D/T_{Control} = 0.31 \pm 0.03$ vs. $D/T_{Rac1} = 0.54 \pm 0.04$; $P < 0.0001$) and a slight decrease in velocity ($V_{Contr} = 31.6 \pm 1.0$ μm/h vs. $V_{Rac1} = 25.5 \pm 1.6$ μm/h; $P < 0.005$).

the tumor cell line U87-MG also showed enhanced directional persistence of migration (Videos 6 and 7, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1) with a 74% increase in the D/T ratio after Rac knockdown (Fig. 4, A and C). The three cell types showed minimal to moderate decreases in migration speed after partial Rac suppression. However, strong reductions in Rac activity resulted in the suppression of lamella formation and the failure of cells to migrate as noted in many other studies (Nobes and Hall, 1995; Allen et al., 1998; Glogauer et al., 2003; Pradip et al., 2003; unpublished data).

Biological mechanism of Rac regulation

The cell biological mechanism underlying this Rac-dependent regulation of migration became apparent from time-lapse video recordings, where each change in the direction of migration was accompanied by a change in the leading edge of the cell: a new lamella elsewhere on the cell became dominant (Fig. 5 A). In contrast, cells with reduced active Rac were more elongated and had lamellae confined to only one or both ends of the cell. We tested the hypothesis that Rac1 regulates peripheral versus such axial lamellae by quantifying these locomotory structures. Staining of F-actin with phalloidin allowed us to identify morphologically distinct, ruffling lamellae (Wu et al., 2003; Fig. S4, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1). The presence of lamellae at an end of the long axis of the cell (within 20° of the longest dimension of each cell) was compared with the number of more peripheral lamellae located outside of this central zone (Fig. 5 B).

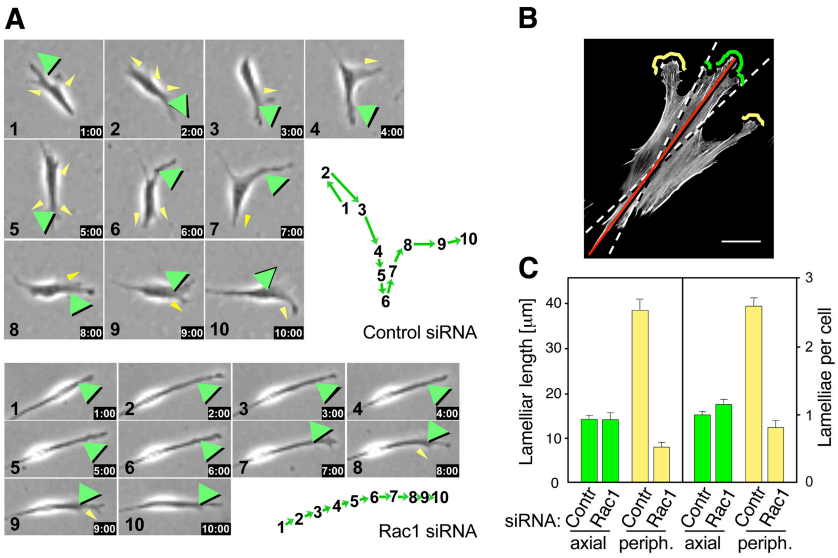

Figure 5. **Suppression of Rac reduces the number of peripheral lamellae and changes in direction of cell migration.** (A) Primary human fibroblasts transfected with control or Rac1 siRNA were recorded by time-lapse video microscopy. Phase-contrast micrographs and migration paths of two representative cells are shown at 1-h intervals. Green arrowheads indicate lamellae extending in the direction of cell movement. Yellow arrowheads show lamellar protrusions in other directions. Green arrows in the diagrams to the right of the micrographs indicate the direction and distance of cell movement each hour. (B and C) Quantification of Rac-dependent lamellae in an axial versus a peripheral location. The main cell axis was determined as the longest distance between the ends of each cell (continuous red line in the image of a cell stained for F-actin). Two zones were defined as follows: "axial" was within 20° of the main axis (area between the dashed white lines) and "peripheral" was anything lateral to this zone. The length of total membrane in all lamellae of each cell and the number of lamellae per cell were determined for axial (green) compared with peripheral (yellow) lamellae in cells transfected with control (Contr) and Rac1 (Rac1) siRNA and presented as mean values ± SEM. Bar, 20 μm.

Knockdown of Rac1 strongly inhibited only the formation of peripheral lamellae. There were threefold fewer peripheral lamellae per cell and a fivefold reduction in lamellar membrane; i.e., the total length of cell membrane associated with these structures per cell (Fig. 5 C). In contrast, there were no detectable effects of Rac reduction on lamellae located at the ends of the long axis of cells, as determined both by the number of lamellae per cell and by the total length of lamella membrane (Fig. 5 C). As a direct consequence of this selective loss of peripheral lamellae, the total number of lamellae per cell was reduced by half, and overall lamella length was decreased by 57% in human fibroblasts with Rac1 knockdown and by 66% in the integrin mutant cells (Table I). Restoration of Rac activity levels in the integrin mutant cells by expression of activated Akt restored normal numbers of lamellae and lamellar length, whereas overexpression of activated Rac1 enhanced numbers of lamellae and length accompanied by an elevation of random motility as reflected by a low D/T ratio (compare Table I and Fig. 1, D and E, with Fig. S1 C and Fig. S3 D). Collectively, these results demonstrate that active Rac induces random motility by promoting peripheral lamellae oriented in directions different from the direction of migration along the main cell axis.

Chemotactic-directed migration versus intrinsic persistence of directional migration

We compared this mechanism of Rac regulation of directional persistence with chemotaxis-induced directed cell migration. Chemotactic migration is known to be regulated by PI3K and localized phosphatidylinositol lipids (Haugh et al., 2000; Srinivasan et al., 2003; Sasaki et al., 2004; Van Haastert and Devreotes, 2004). We confirmed that for human fibroblast chemotaxis toward fMLP (*N*-formyl-Met-Leu-Phe), inhibition of PI3K by LY 294002 with a loss of PIP_3 localization, as detected by a fluorescent pleckstin homology domain probe, inhibited chemotaxis (Fig. 6, A and B). In marked contrast, inhibition of PI3K did not suppress—and, in fact, moderately promoted—intrinsic directionally persistent migration (Fig. 6 D). This increased directionality was associated with a 30–40% decrease in Rac activity (Fig. S2 B). Similar results to those were obtained with U87-MG glioblastoma cells treated with Wortmannin (unpublished data). Importantly, cells exhibiting increased directional persistence of migration because of active Rac1 reduction by siRNA treatment showed no loss of directionality after inhibition of PI3K and loss of PIP_3 localization (Fig. 6 D), even though chemotaxis was blocked. Neither suppression of Rac activity nor treatment with LY 294002 had a significant effect on activation-associated phosphorylation of the MAP kinases ERK1/2 and JNK (Fig. S2). Consistent with previous data establishing a role for Cdc42 in chemotactic-directed migration, suppression of Cdc42 levels and activity by >80% suppressed fibroblast chemotaxis (Fig. 6 C).

Table I. **Rac-dependent changes in lamellae**

Cell line/clone	Lamella length/cell	Lamellae/cell
	μm	
GD25 β_1 wild-type	51.0 ± 2.5	2.8 ± 0.1
GD25 W775A	17.1 ± 1.5	1.6 ± 0.1
GD25 W775A Akt 1	29.9 ± 1.9	2.1 ± 0.1
GD25 W775A Akt 2	53.0 ± 3.9	2.6 ± 0.2
GD25 W775A Akt 3	56.1 ± 2.5	2.8 ± 0.1
GD25 W775A Rac 1	94.1 ± 5.6	3.5 ± 0.1
GD25 W775A Rac 2	105.5 ± 5.0	2.9 ± 0.1
GD25 W775A Rac 3	95.9 ± 4.4	3.4 ± 0.1
HFF control siRNA	52.0 ± 2.0	3.7 ± 0.1
HFF Rac1 siRNA	22.1 ± 2.0	2.0 ± 0.1

The length and number of lamellae per cell were measured for at least 45 cells from each cell line/condition and quantified by image processing software (MetaMorph version 4.6). Values represent mean ± SEM.
HFF, primary human foreskin fibroblasts.

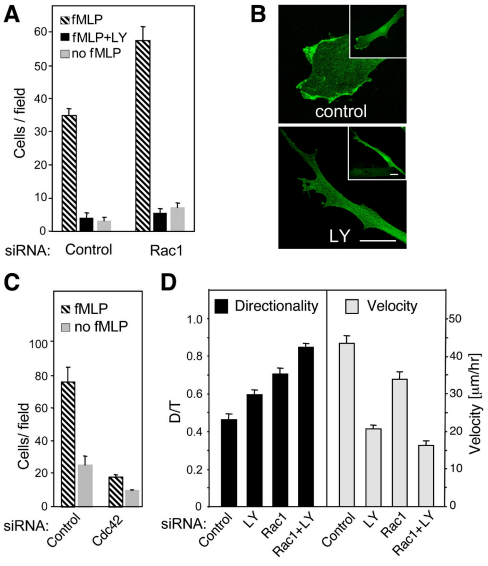

Figure 6. **Inhibition of PI 3-kinase or Cdc42 inhibits chemotaxis but not directionally persistent cell migration.** (A) Inhibition of PI3K by 50 μM LY 294002 blocks primary human fibroblast chemotaxis toward 300 nM fMLP whether or not Rac is suppressed by 1 nM Rac1 siRNA. (B) Localization of GFP-tagged Akt PH domain to lamellae is suppressed by treatment with LY 294002. Insets show lower magnification views of the entire cell. Bars, 20 μm. (C) Inhibition of Cdc42 by 100 nM Cdc42 siRNA (>80% of both total Cdc42 protein and activity) blocks fMLP-stimulated chemotaxis of human fibroblasts. (D) Inhibition of PI3K by 50 μM LY 294002 does not suppress the increased directional persistence of migration induced by reduction of active Rac using 1 nM Rac1 siRNA. (A, C, and D) Error bars represent SEM.

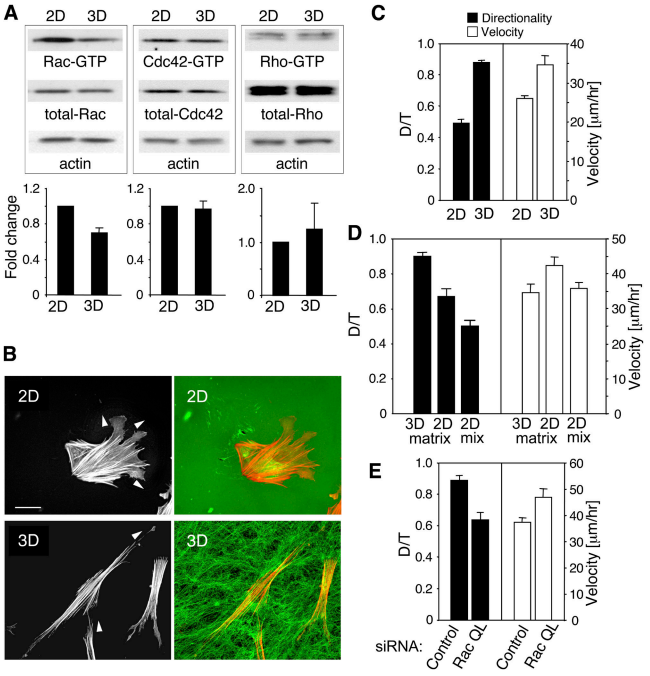

Figure 7. **3D fibronectin matrix reduces both Rac activity and random migration.** (A) Primary human fibroblasts were plated on 2D substrates coated with fibronectin or 3D matrices rich in fibronectin and assayed for activity as in Fig. 1. The reduction of active Rac in cells plated in a 3D matrix compared with 2D was 31% ($P = 0.017$), whereas Cdc42 and Rho changes were not significant (Cdc42 4.5% reduction, $P = 0.66$; Rho 25% increase, $P = 0.65$). Error bars indicate SEM. (B) Cells in 2D versus 3D environments have different morphologies and locations of lamellae (arrowheads), as shown after staining for F-actin with phalloidin-Alexa 594 (left and red color in the right) and anti-fibronectin antibody (green). Bar, 20 μm. (C) Migration of cells in 2D versus 3D environments was recorded, and directionality (D/T) and velocity were calculated. (C and D) Error bars represent SEM. (D) Decreased random motility of cells within three-dimensional (3D) matrix. Primary human fibroblasts were cultured overnight within intact 3D matrices, on mechanically flattened 3D matrix (2D matrix), or on surfaces coated with solubilized 3D matrix (2D mix). Cell movements were recorded for 10 h, and the D/T ratio and velocity were calculated as described above. (E) Activated Rac in cells in 3D matrix restores random motility. Primary human fibroblasts were cotransfected with Rac Q61L VSV and Rac Q61L GFP or GFP alone (Control), sorted for low levels of GFP expression (1–50% from the peak of positive cells), and plated on 3D matrices. Cell movements were recorded for 10 h, and the D/T ratio and velocity were quantified. The bars represent the means of data pooled from two experiments, and error bars indicate SEM (n = 32–34 cells).

Rac and migration in 3D matrix

Cells migrating in 3D cell-derived matrices have different types of cell adhesions, morphology, and signaling when compared with cells in standard 2D tissue culture (Cukierman et al., 2001; Walpita and Hay, 2002). Human fibroblasts in such 3D matrices were found to have a partial, but highly reproducible, 30–50% reduction in active Rac, but no reduction or increase in Cdc42 or Rho activities (Fig. 7 A). This reduction in Rac activity was accompanied by decreased random migration (Fig. 7 C and Video 3), with directional persistence increasing from $D/T_{2D} = 0.48 \pm 0.03$ to $D/T_{3D} = 0.87 \pm 0.02$ ($P < 0.0001$). Individual cells in the 3D matrix became spindle shaped as previously described (Cukierman et al., 2001), but in addition, staining for F-actin revealed a substantial reduction in peripheral lamellae in 3D compared with 2D (Fig. 7 B). In contrast to the results in cells cultured on 2D substrates, there was a significant increase in overall cell migration velocity by 34% associated with the reductions in active Rac and random migration in a 3D environment (Fig. 7 C; $P < 0.0001$). These findings further indicate the separate regulation of speed and directionality.

Because the differences between 2D fibronectin and 3D substrates could have been the result of either three dimensionality or of differences in molecular composition, the 3D matrix was converted to 2D as a direct test of the role of dimensionality. Physically flattening 3D cell-derived matrices by compression still retained the increased overall velocity, but it was accompanied by a restoration of random motility (Fig. 7 D; $P < 0.0001$). Moreover, solubilizing the matrix and spreading its mixed components on a 2D substrate showed the same restoration of random motility (Fig. 7 D; $P < 0.0001$), with migration rates similar to those on 3D matrix (no statistically significant difference). These analyses indicate that the increased directionality in 3D is related to the three dimensionality of the matrix, and that it is unrelated to cell migration speed.

A highly oriented 3D matrix, such as from tumor-derived stromal cells, can affect the orientation and potentially the migration of cells (Amatangelo et al., 2005). Although local parallel alignment and migration of closely adjacent cells was sometimes present in our 3D matrix cultures, there was no general pattern of parallel migration. Persistence of migration occurred in all directions in the 3D matrix (Fig. S5, A and B, available at http://jcb.org/cgi/content/full/jcb.200503152.DC1). Finally, directly

increasing Rac activation levels by transfection with constitutively activated Rac produced an increase in random motility in the 3D matrix with a 30% decrease in the D/T ratio (Fig. 7 E; $P < 0.001$)) and increased lamellae (not depicted), confirming that Rac confers random motility in a 3D as well as in a 2D environment.

Discussion

Our central finding is that a relatively small change in total Rac1 activity can serve as a switch that regulates the overall intrinsic pattern of cell migration of a cell. By using at least three different approaches (mutagenesis, RNA interference, and manipulation of the extracellular environment between 2D and 3D), we demonstrate that moderate levels of active Rac support random motility by selectively promoting peripheral lamellae that permit cell turning, but reductions of active Rac by $\geq$30% instead support directionally persistent migration using axial lamellae.

This role of Rac in regulating the capacity for random versus directionally persistent motility was found for a variety of cell types, including fibroblasts and epithelial cells, suggesting that it is a common phenomenon. Moderate changes in Rac activity did not necessarily affect the velocity of cell migration, and in a 3D environment, suppression of Rac activity occurred together with increased velocity, indicating the independence of directionality from velocity. Primary human fibroblasts express Rac1 and traces of Rac2 and Rac3 (unpublished data); it is possible that reductions in Rac1 were able to produce such large effects on the mode of migration without changes in overall migration velocity because other Rac isoforms or other regulators of migration speed were still present. Unlike velocity, directionality strongly depended on cellular levels of Rac-GTP and not Cdc42 or Rho.

In terms of a cell biological mechanism, slightly lowering Rac1 activity resulted in suppression of peripheral lamellae, which promoted persistent migration of a cell in one direction because of the absence of new peripheral lamellae that could produce a change in the direction of cell migration. This regulation of intrinsic directionality differs markedly from chemotactic-directed migration, which depends on PI3K and localized PIP_3 with Rac or Ras localization to active lamellae (Srinivasan et al., 2003; Sasaki et al., 2004; Van Haastert and Devreotes, 2004). It also differs from mechanisms involving local changes in Rac activity during chemotaxis and α_4 integrin–regulated migration (Arrieumerlou and Meyer, 2005; Nishiya et al., 2005).

In contrast to this novel role of small changes in total Rac activity in regulating the overall pattern of cell migration, substantial increases in Rac activity are well-known stimulators of the velocity of cell migration (Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). Conversely, cells with major deficiencies in Rac as a result of gene ablation or RNA interference are reported to show marked defects in overall cell migration and chemotaxis (Roberts et al., 1999; Glogauer et al., 2003; Sun et al., 2004; Weiss-Haljiti et al., 2004). Rac activation is accompanied by its translocation to the plasma membrane (del Pozo et al., 2002, 2004), with the highest concentrations of active Rac located at the leading edge of motile cells (Kraynov et al., 2000; Itoh et al., 2002; Schlunck et al., 2004). Active Rac in lamellipodia at the leading edge is thought to function by interacting with WAVE to stimulate Arp2/3-mediated actin polymerization, producing lamellar extension and forward cell movement (Miki et al., 2000; Pollard et al., 2000). However, cell migration can still proceed effectively in the absence of lamellipodia (Gupton et al., 2005), suggesting that the target of Rac may be the lamella rather than lamellipodia. We emphasize again that our studies have focused on the role of total or global levels of cellular Rac1 activity as a central regulator, rather than on localized Rac.

Our findings indicate that Rac1 levels regulate persistence of migration by controlling the number of peripheral lamellae and associated total amount of membrane protrusions that can mediate cell turning (3–5-fold difference), whereas the number of axial lamellae and associated protrusive membrane located along the long axis of the cell did not change (1–1.1-fold dfference). The original concept of the dominance of, first, one, and then another lamella during cell migration, which explains the capacity of fibroblasts and other cells to turn and migrate in a new direction, has been known for decades (Trinkaus, 1969). Our study describes a signaling process that limits the number of peripheral lamellae used for turning.

Studies of Rac and other Rho GTPases have traditionally depended heavily on overexpression of constitutively activated and dominant-negative constructs. Although useful for manipulating levels of activity, they could theoretically be accompanied by artifacts because the GTPase does not cycle or the construct might affect an unknown range of regulatory molecules. In fact, we found that strong overexpression of both types of constructs to produce a large suppression or enhancement of Rac activity disrupted cell migration with a substantial decrease in velocity, which is consistent with previous studies (Nobes and Hall, 1995; Allen et al., 1998; Banyard et al., 2000; Glogauer et al., 2003; Pradip et al., 2003). As alternative approaches, our use of an integrin mutant, RNA interference to produce stepwise reductions in total Rac activity, comparisons with other Rho GTPases, testing the effects of physiological changes in the type of substrate on Rac and migration pattern, and the use of a Rac small-molecule inhibitor were designed to provide independent tests of the role of Rac activity in the pattern of cell migration. All of these independent approaches support the central role of the total Rac activity in regulating random versus directionally persistent migration.

As depicted in Fig. 8, we suggest that there may be at least four distinct levels of Rac activity differentially regulating the speed and pattern of intrinsic cell migration. As described by others (e.g., Glogauer et al., 2003), very low levels of active Rac result in immobilization of the cells (Fig. 8, state I). Naturally occurring levels of active Rac higher than this extreme state, but still $\sim$30–50% lower than in cells cultured on a 2D fibronectin substrate, were found in fibroblasts grown in 3D (Fig. 8, state II). Under these conditions, cells retained a spindle-shaped morphology, but developed a stable, single lamella in the direction of migration containing membrane-localized

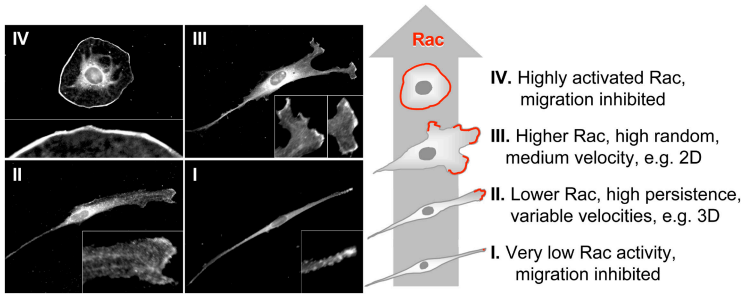

Figure 8. **Four stages of Rac-GTP regulation of cell morphology and types of cell motility.** The amount of Rac localized in lamellae as shown by anti-Rac1 antibody localization (left) and depicted schematically in the diagram (red) with increasing levels of Rac activation (broad gray arrow) from state I to state IV. Increased levels of total cellular active Rac produce characteristic increases in the extent of random cell motility accompanied by varying velocities of cell migration in 2D versus 3D culture or after experimental manipulations of Rac activity. Rac activation that is too low or too high leads to immobilization, with stunted lamellae at low levels and continuous, circumferential lamellae or cell rounding at very high levels (Discussion). The cells shown in the examples were primary human fibroblasts stained for Rac1 after culturing on a 2D substrate (state III) or in a 3D matrix (state II) compared with effects of extensive knockdown of Rac1 using 200 nM Rac siRNA (state I) or overexpression of constitutively activated Rac QL after transient transfection (state IV).

Rac1. These state II cells demonstrated both the highest directionality and highest velocity of migration. We suggest that this condition would be ideal for cell migration in vivo; e.g., during neural crest and myoblast cell migration in embryonic development. Elevation of Rac levels by extrinsic or intrinsic factors could disrupt these processes. In addition, our finding that chemotaxis can be stimulated by a moderate reduction in Rac suggests that the specific level of overall Rac activation may also influence chemotactic efficiency in vivo.

The low random motility of state II cells in 3D could be mimicked by suppressing active Rac in cells in 2D cultures 30–95% below steady-state levels. Levels of active Rac found normally in cells attached to 2D plastic or fibronectin-coated substrates in regular tissue culture promoted the formation of peripheral lamellae and led to an increased random migration (Fig. 8, state III). Random migration could also be induced in state II cells in 3D matrices by the experimental elevation of Rac. At very high levels of Rac-GTP accompanying constitutive activation, cells acquired rounded nonruffling or pancake-shaped, highly ruffled morphologies, and they became immobilized (Fig. 8, state IV). This state might occur during initial cell spreading when cells show transient Rac activation (Price et al., 1998) without migration. The key finding, however, is that relatively small changes in total Rac can serve as a central regulator of intrinsic random versus directionally persistent cell migration.

Materials and methods

Cell culture

Primary human foreskin fibroblasts were a gift from Susan Yamada (National Institute of Dental and Craniofacial Research [NIDCR], Bethesda, MD) and were used at passages 5–18. The GD25 β_1-null fibroblast cell line was a gift from R. Fässler (Max Planck Institute, Martinsried, Germany). GD25 cells expressing wild-type or mutant β_1 integrin were generated as described previously (Pankov et al., 2003). U87-MG human glioblastoma and MCF-10A human breast epithelial cell lines were originally purchased from American Type Culture Collection. All cell lines except MCF-10A were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MCF-10A cells were cultured in DMEM/Ham's F12 medium supplemented with 20 ng/ml EGF, 10 μg/ml insulin, 500 ng/ml hydrocortisone, and 5% equine serum. 3D fibronectin-containing matrices were prepared as described previously (Cukierman et al., 2001).

A 3D matrix on a coverslip was mechanically compressed by applying a weight of 940 g to a central area of 625 mm^2 (1.5 g/mm^2) for 5 min to generate a 2D matrix (Cukierman, 2002). Alternatively, 3D matrices were solubilized in 5 M guanidine containing 10 mM DTT and 5 mM PMSF. The dissolved matrix components were coated on tissue culture dishes at a protein concentration of 50 μg/ml.

Antibodies and reagents

Antibodies to total-, phospho (Ser 473)-, and phospho (Thr 308)-Akt as well as total, phospho (Thr 202), and (Tyr 204) p44/42 MAP kinase (ERK1/2) were purchased from Cell Signaling; anti-Cdc42 antibody was purchased from BD Biosciences; phalloidin conjugated with Alexa 488 or Alexa 594 was obtained from Molecular Probes; total, phospho (Thr 183), and phospho (Tyr 185) JNK antibodies, Cdc42 siRNA sc29256, and RhoA siRNA sc29471 were obtained from Santa Cruz Biotechnology, Inc.; anti-RhoA antibodies were purchased from Santa Cruz Biotechnology, Inc. or Cytoskeleton, Inc.; anti-VSV epitope and antiactin clone AC-40 were purchased from Sigma-Aldrich; anti-Rac antibody and Rhotekin-Rho binding domain were purchased from Upstate Biotechnology or Cytoskeleton, Inc.; anti-β_1 antibody 4080 was raised against a 50-mer cytoplasmic domain peptide in a rabbit; and anti-β_3 integrin hybridoma AP3 was purchased from American Type Culture Collection. PAK1 PBD agarose was purchased from Upstate Biotechnology or Cytoskeleton, Inc. Rac1 siRNA and the nonspecific control siRNA pool were obtained from Dharmacon. The individual sequences (sense) for each Rac1 siRNA duplex were as follows: 5, AGACGGAGCUGUAGGUAAAUU; 7, UAAGGAGAUUGGUGCUGUAUU; 8, UAAAGACACGAUCGAGAAAUU; and 9 (with ON TARGET™ modification for enhanced specificity), CGGCACCACUGUCCCAACAUU. These siRNAs were used either as a pool of all four or as individual siRNA duplexes.

The Rac inhibitor NSC23766 was recently reported (Gao et al., 2004) to be a Rac-specific small-molecule inhibitor that targets Rac activation by GEF. The compound was designed, synthesized, and provided by the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD). LY 294002 was purchased from Calbiochem.

Plasmids

An expression plasmid containing cDNA encoding the small GTPase Rac1 was provided by J. Silvio Gutkind (NIDCR). The constitutively activated Rac1 construct pRK VSV RacQ61L (Rac QL) with a VSV epitope tag was described previously (Koivisto et al., 2004). A cDNA clone encoding Akt was purchased from Upstate Biotechnology. For constitutive activation, the myristoylated vector pRKmyr was constructed by inserting the Kozak consensus sequence and the 13 NH_2-terminal amino acids of c-Src into the pRK5-based expression system. BamHI and XbaI sites were introduced into Akt using PCR, and Akt was subcloned into pRKmyr to obtain pRKmyrAkt. HindIII and SalI sites were introduced by PCR to flank the Akt PH domain (aa 1–148), and the PH domain was subcloned into pEGFP-C1 to generate the pEGFP-C1Akt-PH probe (Kontos et al., 1998; Servant et al., 2000).

siRNA transfection, pull-down assays, and immunoblotting

Primary human fibroblasts were transfected with 0.01–200 nM siRNA using Lipofectamine 2000 (Invitrogen) and OptiMEM medium (GIBCO BRL) in the absence of antibiotics according to the manufacturer's recommendations. Transfected cells were passaged at 56–60 h and used after 72 h. Transfection efficiency as determined by fluorescence detection of Dharmacon siGLO RISC-free siRNA at transfection concentrations ranging from 0.01 to 100 nM was 100% at all of these concentrations tested. Pull-down assays for Rho-GTPases were performed as described previously (Sander et al., 1998) with minor modifications. In brief, cells were scraped into ice-cold lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Igepal, 10% glycerol, and Complete™ protease

cocktail without EDTA [Roche]), and centrifuged for 5 min at 14,000 *g*. Cleared lysates were incubated with 10 or 20 μg PAK-1 PBD agarose or 20 μg GST-tagged Rhotekin Rho-binding domain bound to glutathione agarose for 45 or 60 min at 4°C with rotation. The beads were washed three times with lysis buffer and heated for 3–5 min at 95–100°C in reducing SDS-PAGE sample buffer, and the released proteins were resolved on 8–16% gradient gels (Novex). After electrotransfer to nitrocellulose membranes (Novex), the filters were blocked (5% nonfat dry milk in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 0.1% Tween 20) and probed with the indicated antibodies, followed by the appropriate secondary horseradish peroxidase-conjugated antibodies (Amersham). Immunoblots were visualized using the ECL system and Hyperfilm X-ray film (Amersham Biosciences). For experiments quantifying the effects of different levels of active Rac on the D/T ratio, immunoblots were analyzed by using a chemiluminescent imager (LAS-1000 Pro V3.12; Fuji) after development with the Super Signal West Femto Maximum Sensitivity Substrate (Pierce Chemical Co.); results were quantified using Image Gauge V4.22 software (Fuji). All pairs or sets of gel images used samples that were analyzed on the same polyacrylamide gel.

Microscopy

Cells were plated on tissue culture dishes with or without precoating with 1 or 5 μg/ml human plasma fibronectin or onto 3D fibronectin-containing matrices at a density of 500 cells/cm^2. After overnight incubation, the medium was changed, and cell movements were monitored with microscopes (Axiovert 25; Carl Zeiss MicroImaging, Inc.) using 5× NA 0.12 or 10× NA 0.25 A-Plan objectives. Images were collected with CCD video cameras (model XC-ST50; Sony) at 10- or 15-min intervals, digitized, and stored as image stacks using MetaMorph 6.16 software (Universal Imaging Corp.). Velocity and persistence of migratory directionality (D/T) were determined by tracking the positions of cell nuclei using the Track Point function of MetaMorph. Control experiments indicated that the D/T ratio was the same for the first and second halves of the analysis period (Fig. S5 C, and that the 10–15-min rate of acquisition of images was sufficient to minimize the asymptotic approach to a constant D/T ratio that can occur if sampling was conducted at 1-h intervals (Fig. S5 D). The mean square displacement values were obtained using the Track Object function, which determined the intensity centroids of the defined target regions (i.e., the cells) and tracked their displacement automatically through the planes of each image stack.

Cells for immunofluorescence analysis were plated on uncoated or fibronectin (5 μg/ml) or 3D matrix precoated glass coverslips (12 mm; Carolina Biological Supply Company) and cultured overnight. Samples were fixed with 4% PFA in PBS containing 5% sucrose for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 3 min. Samples were stained with phalloidin that was conjugated with Alexa 488 or Alexa 594 as recommended by the manufacturer. Stained samples were mounted in GEL/MOUNT (Biomeda Corp.) containing 1 mg/ml 1,4-phenylendiamine (Fluka) to reduce photobleaching. Immunofluorescence images were obtained using 40× NA 1.0 or 63× NA 1.5 oil immersion objectives of a microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera (CoolSNAP ES; Photometrics). Digital images were obtained using MetaMorph 6.16 software. Each figure shown is representative of a minimum of two to three independent experiments analyzing 30–45 cells each.

Chemotaxis assay

Primary human fibroblasts were detached with trypsin-EDTA, rinsed, and preincubated with 50 μM LY 294002 (Calbiochem) or vehicle (0.2% ethanol) in complete medium for 15 min in suspension before plating. Cells (8×10^4) were plated onto a Transwell system insert (8-μm pore size; BD Falcon) in a 6-well plate and placed in 3 ml of complete medium with or without 300 nM fMLP (Sigma-Aldrich), LY 294002, or vehicle. Cells were incubated at 37°C for 2 h, and then the inserts were washed with PBS and fixed with 2% PFA in PBS. Cells were removed from the top part of the membrane, and the cells that had migrated to the bottom of the membrane were counted using a phase-contrast microscope. The mean number of cells per field ± SEM of five randomly chosen fields was calculated for each condition.

Online supplemental material

Fig. S1 shows directionally persistent migration of mutant integrin cells and suppression by expression of active Rac. Fig. S2 provides examples of loss of both Rac protein and active Rac (Rac-GTP) after treatment with Rac siRNA without effects on activation-associated phosphorylation of Akt, ERK, or JNK; it also confirms activity of the PI 3′-kinase inhibitor LY 294002. Fig. S3 shows the effects on cell migration of Rac1 knockdown by Rac1 siRNA duplexes or Rac1 overexpression. Fig. S4 shows the lack of effects of Rac1 knockdown on integrins and morphology of cells with an integrin mutation or Rac1 knockdown. Fig. S5 shows analysis of cell migrations in 2D and 3D environments by time-lapse recording.

All videos show time-lapse video recordings at 10-min intervals for 12 h and were recorded using a 5× objective except for video 3, which uses a 10× objective to show cell interactions with the pliable, randomly oriented 3D matrix. Video 1 shows human fibroblasts migrating after transfection with control siRNA. Video 2 shows human fibroblasts migrating after transfection with 0.1 nM Rac1 siRNA. Video 3 shows human fibroblasts migrating within a 3D matrix. Video 4 shows MCF-10A human epithelial cells migrating after transfection with control siRNA. Video 5 shows MCF-10A cells migrating after transfection with 50 nM Rac1 siRNA. Video 6 shows U87-MG human glioblastoma cells migrating after transfection with control siRNA. Video 7 shows U87-MG migrating after transfection with 50 nM Rac1 siRNA. Online supplemental material is available at http://jcb.org/cgi/content/full/jcb.200503152.DC1.

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