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Control of the Direction of Lamellipodia Extension through Changes in the Balance between Rac and Rho Activities

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Abstract: The direction in which cells extend new motile processes, such as lamellipodia and filopodia, can be controlled by altering the geometry of extracellular matrix adhesive islands on which individual cells are cultured, thereby altering mechanical interactions between cells and the adhesive substrate [Parker (2002)]. Here we specifically investigate the intracellular molecular signals that mediate the mechanism by which cells selectively extend these processes from the corners of polygonal-shaped adhesive islands. Constitutive activation of the small GTPase Rac within cells cultured on square-shaped islands of fibronectin resulted in the elimination of preferential extension from corners. This loss of directionality was accompanied by a re-distribution of focal adhesions: the large focal adhesions normally found within the corner regions of square cells were lost and replaced by many smaller focal contacts that were distributed along the entire cell perimeter. Inhibition of the small GTPase, Rho, using C3 exoenzyme blocked lamellipodia extension entirely. However, inhibition of Rho signaling in combination with ectopic Rac activation rescued the corner localization of motile processes and focal adhesions. These results suggest that the ability of cells to sense their physical surroundings and respond by moving in a spatially oriented manner is mediated by a balance between Rho and Rac activities.

keyword: Rac, Rho, Lamellipodia, Motility, Extracellular matrix, Micropattern

1 Introduction

Directional cell migration on extracellular matrix (ECM) is central to numerous physiological processes including development, wound healing and angiogenesis. Individual cells determine their direction of motion based on the position in which they extend new motile processes, such as lamellipodia and filopodia, along their periphery. Although much is known about the signaling molecules that contribute to formation of these motile processes, the mechanism by which cells control the spatial location where these processes will form remains unclear.

By culturing cells on microfabricated ECM islands with defined geometry on the micrometer scale, we have previously demonstrated that the direction in which cells extend new lamellipodia, filipodia and microspikes can be influenced by the global shape of the cell [Parker (2002); Brock (2003)]. For example, when individual endothelial cells, fibroblasts or muscle cells are cultured on square, rectangular, hexagonal, pentagonal, or other angulated geometric ECM islands, they reproducibly extend motile processes from their corners when stimulated with soluble motogens, such as PDGF or FGF. In contrast, round cells on circular islands do not display any directional bias [Parker (2002); Brock (2003)]. Subsequent experiments confirmed that the orientation of these motile processes governs the direction in which the cell will translocate across the substrate when the microfabricated adhesive barriers are removed [Jiang, 2003]. In cells cultured on polygonal ECM islands, focal adhesions assembled specifically in the corner regions, directly behind the sites where lamellipodia are generated, and precisely where cells apply the greatest traction forces on the ECM substrate [Parker (2002); Wang (2002); Brock (2003)]. Separate studies on flexible substrates that differ in their mechanical compliance similarly show that cells move in the direction in which they exert greatest traction forces [Lo (2000)]. Thus, these microfabricated ECM islands provide a simplified experimental system to study the mechanism by which mechanical interactions between cells and ECM govern directional motility.

In the present study, we set out to examine the intracellular molecular signals that mediate the spatial positioning of motile processes in response to physical adhesive interactions between cells and ECM. In particular, we were

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interested in determining how the actions of Rac and Rho are influenced by ECM geometry. These two members of the Ras superfamily of small GTPases have been shown to play a central role in control of cell motility by linking membrane receptors to actin assembly, focal adhesion formation, and cytoskeletal tension generation [Ridley (1992a); Ridley (1992b)]. In general, Rac is thought to regulate the formation of lamellipodia and membrane ruffles, whereas Rho controls tension generation, stress fiber formation and focal adhesion assembly. Many studies also have demonstrated crosstalk between Rac and Rho, and some suggest that Rac and Rho may be mutually antagonistic [Sanders (1999); Edwards (1999)]. Rac may inhibit Rho activity by enhancing production of oxygen radicals that inhibit the tyrosine phosphatase that, in turn, activates the Rho inhibitor, p190RhoGAP, by increasing its phosphorylation [Nimnual (2003)]. Other studies have shown that RhoG activates Rac through its interaction with ELMO, which in turn binds and activates the Rac GEF Dock180 [Katoh (2003); Gauthier-Rouiere (1998)].

Given the extensive evidence suggesting that Rac and Rho are critical mediators of cell motility induced by soluble factors, we therefore set out to address the question: Does Rac, alone or in combination with Rho, contribute to the mechanism by which cells exert *spatial control* over lamellipodia extension, in particular, in response to physical cues from the ECM?

2 Methods

2.1 Experimental System

Our microcontact printing method for producing glass substrates containing micrometer-sized ECM islands has been published previously [Singhvi (1994); Chen (1997); Chen (2000)]. NIH3T3 fibroblasts were passaged in Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% FBS, 4.5 g/L glucose, 2 mM glutamine, 20 mM HEPES, 100 U/mL streptomycin and 100 U/mL penicillin at 37 ^{o}C in 5% CO_2. Cells were serum-starved 1-2 days prior to experiments and plated on micropatterned ECM substrates in NIH3T3 Defined Medium composed of DMEM containing 1% BSA, 4.5 g/mL glucose, 2 mM glutamine and 100 U/mL and 100 U/mL penicillin. Human microvascular endothelial cells from neonate skin were cultured in EGM-2 media (Cascade Biologics, Portland, OR) containing 5% FBS and growth factors (VEGF, IGF, b-FGF) according to the manufacturer's instructions. The Rho-associated kinase (ROCK) inhibitor, Y27632 (Calbiochem) was used at a concentration of 20 μM that produced optimal and reversible effects, as we previously demonstrated with human endothelial cells [Mammoto, 2004]. The Rho inhibitor, C3 bacterial exoenzyme, was purchased from Cytoskeleleton, Inc.

2.2 Synthesis of recombinant proteins

Mutant forms of the small GTPases, RacV12 (constitutively active), RacN17 (dominant negative), RhoV14 (constitutively active), and a dominant negative truncated form of the downstream Rho-effector mDia, were expressed from the *E. coli* expression plasmid pGEX4T that was kindly provided by Alan Hall (University College London, UK). The plasmid encoding full-length mDia was kindly provided by Yoshimi Takai (Osaka University, Japan). The dominant negative form of mDia (mDia978, 543-978 aa) was constructed by PCR using the full-length mDia as template and subcloning into the glutathione-S-transferase (GST)-fusion protein/ *E.coli* expression vector pGEX4T-1 (Amersham Pharmacia, Piscataway, NJ) at the EcoRI/XhoI site [Mammoto (2004)]. Recombinant proteins were produced and purified using the protocol developed by Alan Hall [Self (1995)]. This procedure relies on maintaining a high [Mg2+] in the sample buffers to stabilize the nucleotide-bound form of the GTPases.

Purified recombinant proteins were introduced into cells with the BioPorter protein transfection reagent (Gene Therapy Systems, Inc., San Diego, CA), as previously described [Mammoto (2004)]; greater than 90% expression efficiency is obtained with this method. During the proteofection, the cells were incubated 4 hrs at 37^o C in serum-free DMEM; the medium was then replaced with DMEM containing 1% BSA and the cells were incubated for an additional 2 hrs to permit the release of captured protein from endosomes into the cytoplasm prior to experimental manipulation.

2.3 Immunofluorescence microscopy

For immunostaining studies, cells were fixed in 4% paraformaldehyde in PBS for 10 min and then permeabilized in 0.3% TritonX-100 in PBS for 5 min and washed three times with PBS containing 0.1% BSA, except for staining of membrane receptors which was carried out in non-permeabilized cells. We used and monoclonal

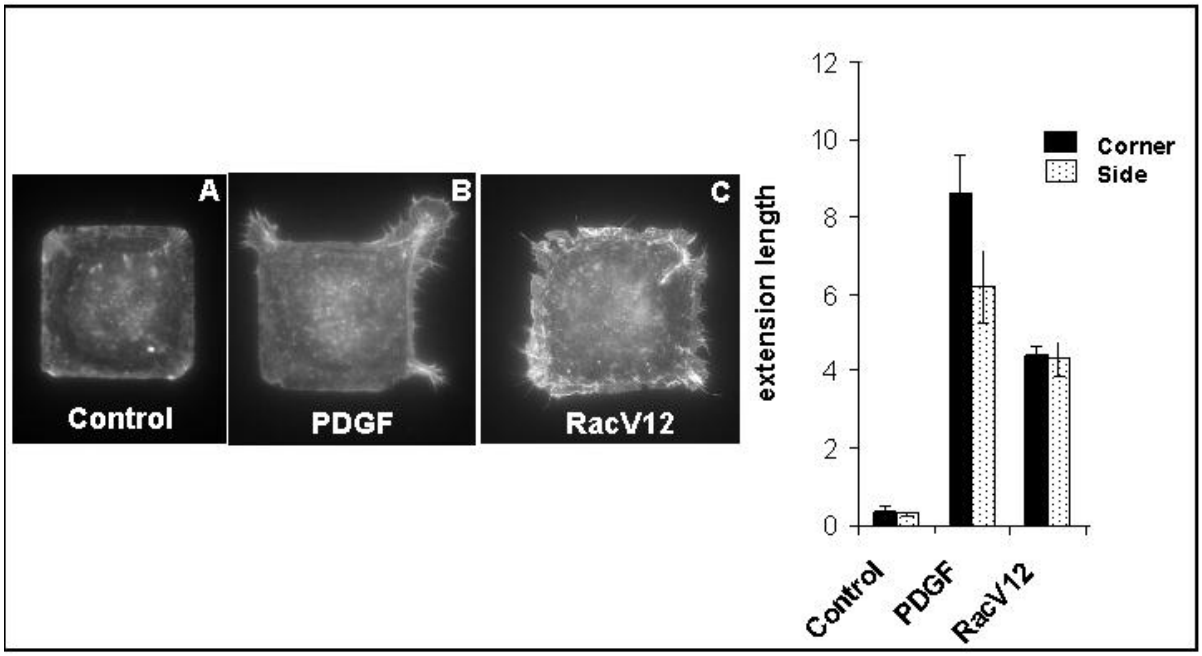

Figure 1 : Square NIH3T3 cells stimulated with PDGF (5 ng/ml) preferentially extended lamellipodia from their corners versus their sides in wild type Rac cells. Constitutive activation of Rac (RacV12) results in equal extension of small ruffles and lamellipodia at all points along the cell perimeter.

antibodies to vinculin (Sigma) and paxillin (Transduction Laboratories). Primary antibodies were detected with secondary antibodies to rabbit, mouse or goat conjugated to Alexa568 or Alexa488 fluorophores (Molecular Probes, Eugene, OR). In certain studies, immunostained cells were simultaneously co-stained with 1:200 Alexa488-conjugated phalloidin (Molecular Probes, Eugene, Oregon) to label actin filaments. Phase and fluorescent images were collected with a Nikon Diaphot inverted microscope.

2.4 Quantitation of focal adhesions

IPLab Spectrum software was used to create scripts for the automated quantitation of total focal adhesion intensity in different regions of the cell. As in the methods previously used to quantitate area of lamellipodia [Parker, 2002], a grid was overlaid on each cell image using the border of the immunostained fibronectin-coated island as a reference point and the grid was used to designate the regions of sides and corners. Grayscale images were normalized, segmented and the mean intensity of total focal adhesion-associated immunofluorescence staining was computed in each region of the grid.

3 Results

3.1 Role of Rac in directional lamellipodia extension

Serum-starved fibroblasts plated on fibronectin-coated square adhesive islands created with microcontact printing spread and remain confined to the shape of the square island (Fig. 1 A) [Chen, 1997]. Upon stimulation with PDGF (5 ng/ml), these square cells preferentially extended lamellipodia from their corners (Fig. 1B), as previously described [Parker (2002), Brock (2003)]. In square cells stimulated with PDGF, the mean length of lamellipodia in the corner regions was 43% greater than that of the processes that spread out from the cell's sides (Fig. 1, right). As expected based on previous reports [Olson, 1995], Rac activity was required for the general process of lamellipodia extension in these NIH 3T3 cells: proteofection of these cells with a dominant negative mutant of Rac (RacN17) eliminated all lamellipodia when cultured on standard culture substrates (Fig. 2).

To determine whether Rac also contributes to spatial control of lamellipodia positioning, cells cultured on square fibronectin islands were proteofected with constitutively active RacV12. This resulted in a loss of the normal spatial constraints such that multiple small lamellipodia and

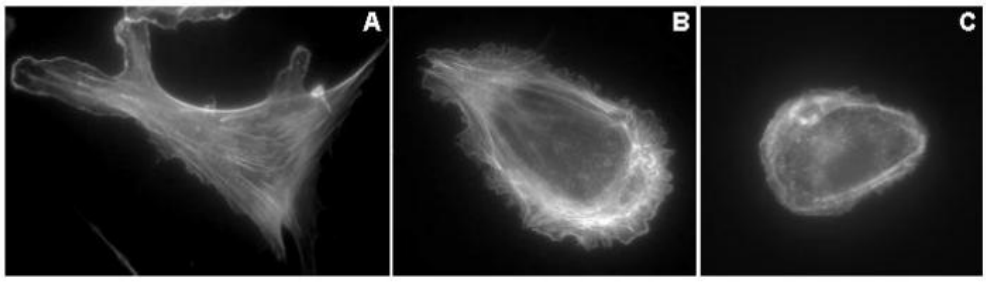

Figure 2 : NIH3T3 wild type cells (A) and similar cells transfected with constitutively active RacV12 (B) or dominant negative RacN17 (C) and stained for F-actin structure with Alexa488-phalloidin. RacV12 cells exhibited increased lamellipodia and membrane ruffles compared to control cells. These motile structures are completely inhibited in cells expressing RacN17 and the total cell area is reduced.

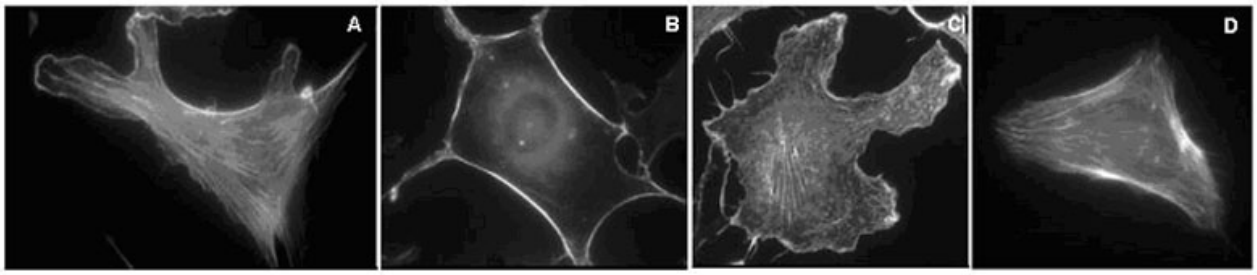

Figure 3 : Rho GTPases regulate actin organizationin NIH3T3 cells as visualized using fluorescent phalloidin staining. Actin stress fibers present within control cells (A) diassemble when Rho signaling is inhibited using C3 exoenzyme (B). Inhibition of ROCK with Y27632 (20 μM) inhibits central stress fiber formation, while increasing actin containing lamellipodia formation along the cell periphery (C). Proteofection of cells with dominant negative mDiaDN partially inhibited stress fiber formation, however, both cells and their motile process were less elongated (D).

ruffles emanated from all points along the perimeter of the square cell (Fig. 1C). The mean lamellipodia length was nearly identical ($\sim$4.4 μm) at the corners and sides in RacV12-expressing cells, compared with average lengths of 8.82 μm + 0.95 μm at the corners and 6.18 + 0.97 μm at the sides in control cells (Fig. 1, right). Thus, constitutive activation of Rac eliminated the bias for the corner localization of motile processes in these polygonal cells, and hence blocked normal spatial control of directional lamellipodia extension.

3.2 Contribution of Rho to directional lamellipodia formation

Interactions between Rac and Rho appear to be critical for coordination of cell tension generation and lamellipodia extension [Ridley (1992); Sanders (1999)]. The effects of Rho on these events are mediated by its two major downstream effectors – ROCK which inhibits myosin phosphatase and results in increased actomyosin-based tension generation [Nakano (1999)], and mDia1, an activator of profilin which stimulates actin polymerization [Wantabe (1999); Li (2003)]. To determine the contribution of Rho and its effectors to directional extension of lamellipodia, cells cultured on square fibronectin islands were either proteofected with constitutively active Rho protein (RhoV14) or inhibitory C3 transferase. ROCK was inhibited using Y27632, a specific small molecule inhibitor, and mDia1 was suppressed by proteofecting cells with a dominant negative mDia GST-fusion protein (mDiaDN) [Mammoto (2004)].

Inhibition of Rho within NIH 3T3 fibroblasts with C3 transferase resulted in the disappearance of stress fibers and a failure to extend lamellipodia (Fig. 3A and Fig. 4A, as previously observed in other cell types [Mam-

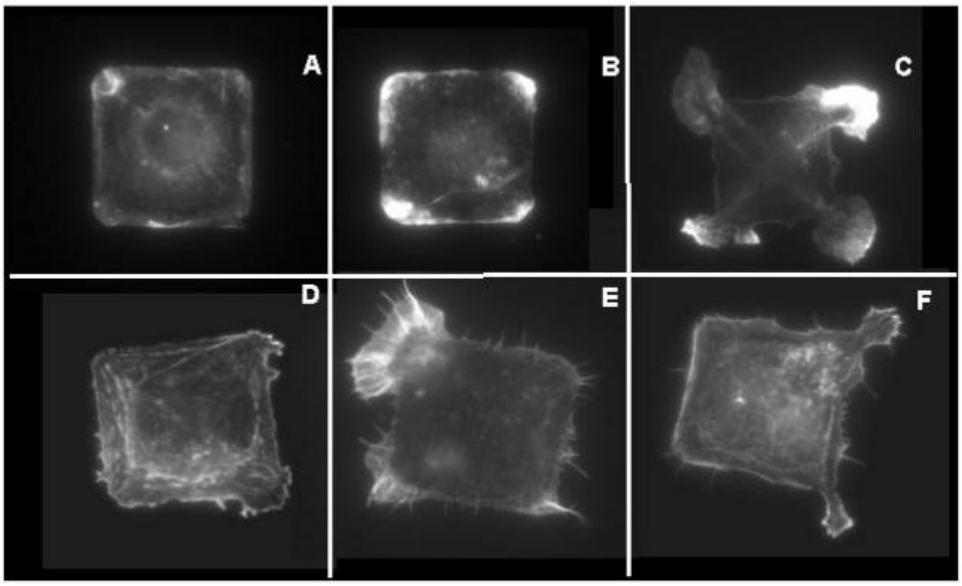

Figure 4 : Downregulation of Rho or its downstream effectors alters actin organization and lamellipodia extension in cells cultured on square ECM adhesive islands, as shown in cells stained with Alexa488- phalloidin. A) Transfection with C3 transferase eliminated all lamellipodia and stress fibers. B) The ROCK inhibitor Y27632 (20 μM) eliminated stress fibers, however, bright peripheral actin staining persisted in the corner regions. C) Dominant negative mDiaDN did not block lamellipodia extension or their localization to corners, even though it inhibited stress fiber formation. C3 transferase (D), dominant negative mDia (F), and treatment with Y27632 (E) restored the normal corner localization pattern of lamellipodia extension when co-proteofected into cells with RacV12.

moto (2004); others]. Y27632 treatment also disrupted stress fibers (Fig. 3B) and decreased lamellipodia extension; however, these cells retained bright peripheral actin staining concentrated in regions at the cell corners where lamellipodia would normally form (Fig. 4B). In past studies, we found that when square cells that already had extended lamellipodia from their corners were treated with Y27632, the lamellipodia became destabilized and collapsed in the corners of the cell [Parker (2002)]. Thus, these data suggest that while ROCK may be required for maintenance of the shape stability of the lamellipodium once it is formed, it is not required for spatial positioning of the site where actin polymerization will be initiated, at least after focal adhesions have already formed.

Proteofection of cells with mDiaDN also did not interfere with preferential extension of lamellipodia from the corners of these cells, although these lamellipodia were broader and stress fibers in the cell body were slightly reduced (Fig. 3D and 4F). We conclude then that mDia is not required for either the spatial localization of lamellipodia or the generation of these motile processes in square cells on micropatterned substrates.

3.3 Antagonism between Rac and Rho signaling

We then asked whether the relative levels of Rac and Rho activities were important for orienting focal adhesions and localizing the motile response. In these studies, cells cultured on microfabricated square adhesive islands were transfected with RacV12 protein in combination with C3 transferase, dominant negative mDia, or in the presence of Y27632. Surprisingly, down-regulation of Rho, or of its downstream mediators ROCK or mDia, resulted in restoration of the normal corner localization pattern of lamellipodia extension in cells expressing constitutively active RacV12 (Fig. 4D-F). Quantitation of these results revealed that inhibition of Rho or coexpression of dominant negative mDia in RacV12-expressing cells restored the normal differences in lamellipodia length between corners and sides observed in control cells (Fig. 5). RacV12 cells treated with the ROCK-inhibitor Y27632 displayed an overall decrease in the length of lamellipodia extension.

However, a statistically significant preference for corner extension was again observed (Fig. 5). The fact that all three perturbations of the Rho pathway could rescue the

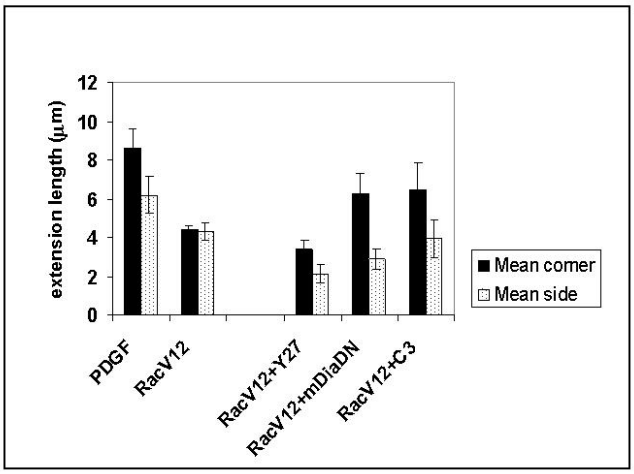

Figure 5 : Wild type cells cultured on individual square adhesive islands and stimulated with the motile agonist PDGF preferentially extended lamellipodia from their corners. Constitutive activation of Rac (RacV12) resulted in loss of this spatial preference, as small ruffles and lamellipodia extended equally from all points along the cell perimeter. Inhibiting Rho or one of its downstream effectors (ROCK or mDia) in cells with ectopically active RacV12, rescued the corner localization pattern (n=30 cells per condition).

localization pattern of lamellipodia extension in cells expressing constitutively active Rac suggests that a balance of Rho and Rac signaling is critical for the cellular response to physical cues from ECM that establish the position where lamellipodia will form and hence, the direction of cell motility.

3.4 Role of Focal Adhesion Positioning

As previously demonstrated [Parker (2002)], large elongated focal adhesions can be detected by immunofluorescence microscopy in the region just behind the corner of each square cells (Fig. 6A), where the cell applies the greatest traction forces to the ECM [Wang, 2002]. Cells that are deficient in the focal adhesion protein vinculin also are unable to extend lamellipodia in response to microinjection with constitutively active Rac, and this response can be reversed by restoring vinculin protein [Goldmann (2002)]. Moreover, vinculin binds the protein ARP 2/3 that helps to initiate the actin polymerization reaction that drives lamellipodia extension [DeMali, (2002)]. We therefore explored whether the altered localization pattern of cell extensions in RacV12 cells might result from a change in the distribution of focal adhesions.

Cells cultured on fibronectin substrates and proteofected with RacV12 lost the normal elongated focal adhesion in their corners, and instead displayed multiple smaller dots containing the focal adhesion proteins, vinculin (Fig. 6B) and paxillin distributed along the entire cell periphery. This dot-like staining pattern is reminiscent of nascent focal complexes previously described in other cells [Riveline (2001)]. Computerized morphometric analysis of these results demonstrated that Rac V12 expression also resulted in a significant decrease in the mean length of both vinculin-and paxillin-containing focal adhesions relative to those in wild type cells (Fig. 6E). While the mean fluorescence staining intensity of paxillin- and vinculin-positive focal adhesions in the corners was also 2.2 times higher than at the sides of control cells, there was no significant difference in intensity of either focal adhesion protein between these two locations in cells expressing RacV12 (Fig. 6E). Thus, RacV12 completely eliminated the bias for focal adhesions to localize in the corners of square cells.

Surprisingly, inhibition of Rho by transfection of RacV12-expressing cells with C3 transferase rescued the bias for the distribution of adhesions to the corner regions (Fig. 6D) with the mean fluorescence intensity of paxillin-positive focal adhesions being almost two times higher at the corners versus the sides (Fig. 6E). Inhibition of ROCK with Y27632 or of mDia with mDia978 in RacV12-expressing cells had a similar effect. Therefore, we conclude that inhibition of Rho in combination with ectopic activation of Rac rescues the corner localization patterns of focal adhesions, and that this correlates directly with the preferential formation of lamellipodia in the same corner regions of these polygonal cells.

4 Discussion

Microfabricated cell adhesive islands permit one to analyze structural changes at sites of lamellipodia extension before they form because the cells consistently generate new motile processes at their corners. Using this novel experimental system, we showed that constitutive activation of Rac (through proteofection with RacV12 protein) causes focal adhesions in square cells to redistribute to positions along the entire cell perimeter, rather

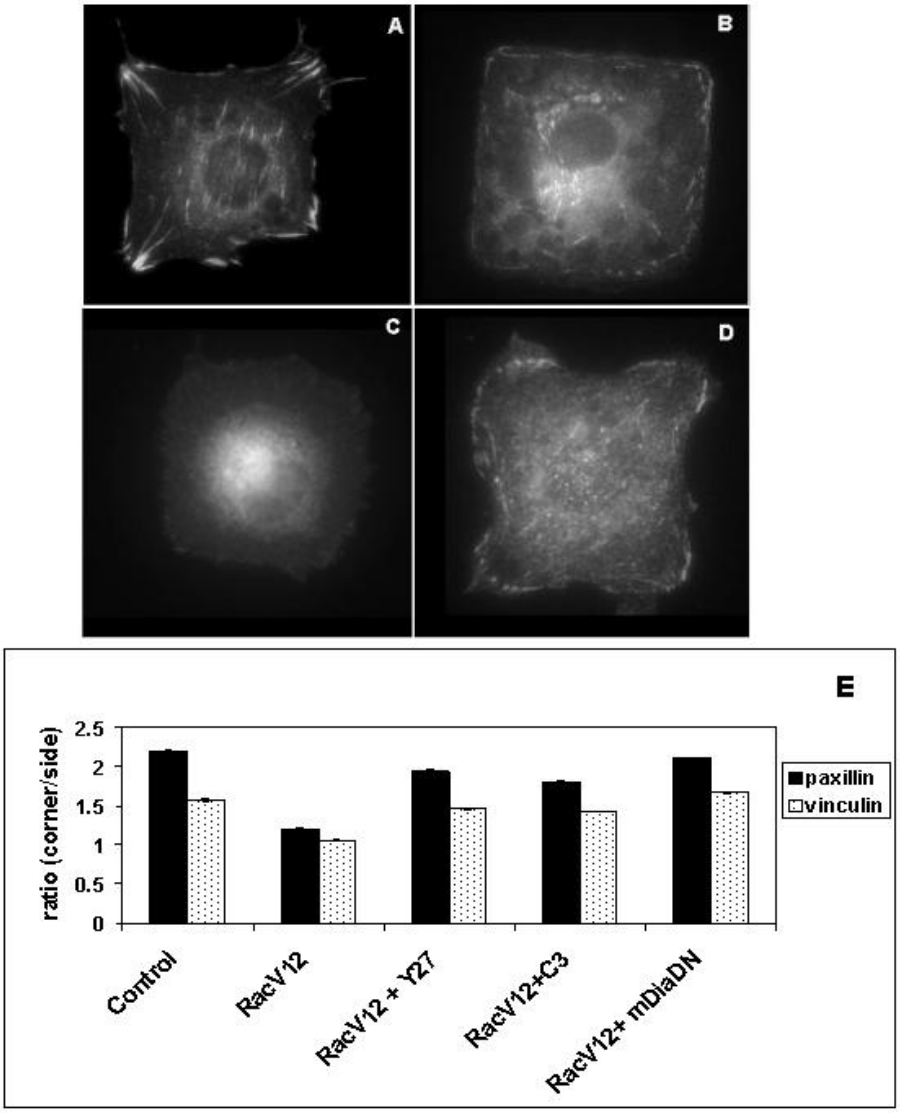

Figure 6 : Inhibition of Rho in combination with ectopic activation of Rac rescues the corner localization of focal adhesions. A) Well developed vinculin-containing focal adhesions preferentially localize in the corners of square control cells. B) Expression of RacV12 results in formation of multiple smaller dot-like vinculin-containing focal adhesions along the entire cell perimeter. C) Inhibition of ROCK with Y27632 caused focal adhesions to disassemble so that only very small structures which stained faintly for vinculin remained. D) Inhibition of Rho by transfection with C3 transferase in cells expressing constitutively active RacV12 restored focal adhesion formation in the corner regions. E) Graph showing ratio of focal adhesion staining between corners versus sides within cells on square islands. Data were quantitated from cells immunostained for two different focal adhesion proteins, vinculin and paxillin, under the various indicated experimental conditions (N=300 adhesions per condition).

than being localized specifically to the area behind the cell corners where lamellipodia normally form. Importantly, the absence of this localization of focal adhesions to the corners was accompanied by a loss of preferential extension of new lamellipodia from these regions. Moreover, treatment of these cells with inhibitors of Rho signaling restored both focal adhesion position and directional extension of new motile process from the corner regions. Previous work from our laboratory has shown that vinculin-containing focal adhesions are required in

order for activated Rac to stimulate lamellipodia formation [Goldmann, 2002]. The studies described here go further and show that the position of focal adhesions also governs *where* Rac manifests its downstream signaling effects that drive lamellipodia extension. Taken together, we therefore conclude that the positioning of focal adhesions in response to physical cues from ECM is critical for determining the site of lamellipodia formation, and that the positioning of these events is controlled through interplay between Rac and Rho.

If the positioning of focal adhesions is the critical process that defines where lamellipodia will form, we then need to ask: What is the mechanism by which ectopic activation of Rac alters focal adhesion distribution? In these studies, we observed that, in addition to displaying a uniform distribution along the cell perimeter, adhesions in RacV12 cells were smaller and morphologically distinct from those observed in control cells. This could result from several types of alterations in the dynamics of focal adhesion turnover. RacV12 may increase the rate of disassembly of existing adhesions, promote formation of focal contacts at new sites, or inhibit the maturation of nascent focal complexes. In addition, some combination of these mechanisms may be acting simultaneously, and this may be mediated by a relative inhibition of Rho-based tension generation that normally facilitates the focal adhesion maturation process [Riveline, 2001; Lele et al, 2005].

In our studies, cells were allowed to spread on square ECM islands and assemble focal adhesions in the corners before being proteofected with RacV12 protein. This argues that the pre-existing large focal adhesions (as observed in the corners of control cells) were either completely or partially disassembled as a result of RacV12 expression, and that the new, smaller adhesions that replace them may be unable to mature into elongated fibrillar structures. Future studies will be required to explore the dynamics of focal adhesion disassembly and formation in square cells expressing constitutively active Rac. In particular, time-lapse imaging of GFP-labeled adhesions may permit the visualization of this process in real time. It is clear however, that the balance between Rac and Rho signaling is critical for the directional extension of lamellipodia. In this work, inhibition of Rho and ROCK signaling had opposite effects in the presence or absence of RacV12. Without RacV12, Rho inhibition blocked lamellipodia extension and disassembled corner focal adhesions, in agreement with the literature [Wantabe (2000); Rottner (1999)]. However, when Rac was constitutively activated, Rho or ROCK inhibition disassembled focal contacts along the sides, and restored both focal adhesion formation and lamellipodia extension at the corners.

The finding that inhibition of Rho or ROCK disassembles focal adhesions and cell protrusions at the sides of the square RacV12-expressing cells is consistent with the effects we and others have observed in control cells [Wantabe (2000); Rottner (1999)]. Why inhibition of Rho or ROCK promotes (i.e., rather than inhibits) focal adhesion formation and lamellipodia extension in the corners of Rac12-expressing cells remains less clear. However, it is interesting to note that ROCK inhibition also promotes formation of focal adhesions in fibroblasts isolated from focal adhesion kinase (FAK) knockout mice [Chen (2002)]. Thus, one possibility is that the remaining focal adhesions in the corners of square RacV12-expressing cells may have less FAK protein than the newly formed focal contacts along the sides, and hence respond locally to Rho or ROCK inhibition in this unusual manner by promoting focal adhesion assembly in these corner regions. Preferential elongation of these adhesions may then preferentially constrain the lamellipodia signaling machinery to these same sites through interactions with ARP2/3 [DeMali, (2002)] or other molecules that are required for the actin polymerization that drives motile process formation.

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