Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels

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Summary

To understand how cells sense and adapt to mechanical stress, we applied tensional forces to magnetic microbeads bound to cell-surface integrin receptors and measured changes in bead displacement with sub-micrometer resolution using optical microscopy. Cells exhibited four types of mechanical responses: (1) an immediate viscoelastic response; (2) early adaptive behavior characterized by pulse-to-pulse attenuation in response to oscillatory forces; (3) later adaptive cell stiffening with sustained (>15 second) static stresses; and (4) a large-scale repositioning response with prolonged (>1 minute) stress. Importantly, these adaptation responses differed biochemically. The immediate and early responses were affected by chemically dissipating cytoskeletal prestress (isometric tension), whereas the later adaptive response was not. The repositioning response was prevented by inhibiting tension through interference with Rho signaling, similar to the case of the immediate and early responses, but it was also prevented by blocking mechanosensitive ion channels or by inhibiting Src tyrosine kinases. All adaptive responses were suppressed by cooling cells to 4°C to slow biochemical remodeling. Thus, cells use multiple mechanisms to sense and respond to static and dynamic changes in the level of mechanical stress applied to integrins.

Key words: Integrin, Focal adhesion, Mechanotransduction, Prestress, Tension, Magnetometry

Introduction

Mechanical stresses influence cell shape and cytoskeletal structure (Wang et al., 1993; Ingber, 1997; Maniotis et al., 1997; Wille et al., 2004), and thereby contribute to the control of many cell behaviors that are crucial for tissue development, including migration, growth, differentiation, apoptosis and stem cell lineage switching (Ingber and Folkman, 1989; Singhvi et al., 1994; Bohmer et al., 1996; Chen et al., 1997; Pelham and Wang, 1997; Parker et al., 2002; McBeath et al., 2004; Moore et al., 2005). Many of these stresses are either transmitted to cells from the extracellular matrix (ECM) or are generated within the contractile cytoskeleton of individual cells and exerted on their ECM adhesions (Ingber, 1997; Geiger and Bershadsky, 2001). Both types of mechanical forces converge on transmembrane integrin receptors that cluster together within specialized focal adhesion sites and physically link the ECM to the cytoskeleton (Wang et al., 1993; Choquet et al., 1997; Ingber, 1997; Geiger et al., 2001).

Cells mechanically adapt to forces applied to integrins or to increases in ECM rigidity by producing a stress-induced strengthening response, and the mechanical stiffness of the cell increases linearly as the level of applied stress is raised (Wang et al., 1993; Choquet et al., 1997; Galbraith and Sheetz, 1999; Giannone et al., 2003; Polte et al., 2004). This enhances the ability of the cell to resist mechanical injury (e.g. prevent membrane tearing) (Glogauer et al., 1998; Wang et al., 2001), and supports cell locomotion by increasing the level of traction forces that cells can exert on their ECM adhesions (Choquet et al., 1997; Pelham and Wang, 1997; Parker et al., 2002; Giannone et al., 2003). Studies using ligand-coated pipettes pulled with a micromanipulator (Riveline et al., 2001), ECM-coated microbeads tensed with an optical tweezer (Choquet et al., 1997; Felsenfeld et al., 1999; Giannone et al., 2003) or similarly coated magnetic microbeads displaced using applied magnetic fields (Matthews et al., 2004b) have revealed that the cell's ability to strengthen itself in response to a sustained applied force requires focal adhesion assembly at the site of force application. These changes in focal adhesion size, composition, and position all occur within the first few seconds to minutes after external stresses are applied to the cell (Choquet et al., 1997; Riveline et al., 2001). On the other hand, when similar mechanical forces are applied to integrins through non-activating antibodies that do not ligate the RGD binding site of the receptor, neither focal adhesion formation (Miyamoto et al., 1995b) nor cell strengthening occur (Choquet et al., 1997; Matthews et al., 2004b).

Force-induced assembly of focal adhesions is mediated by activation of the small GTPase Rho and its downstream targets, Rho-associated kinase (ROCK) and mDia (Riveline et al., 2001). ROCK promotes focal adhesion formation by

promoting myosin light chain (MLC) phosphorylation (via inhibition of MLC phosphatase) (Kimura et al., 1996) and increasing cell contractility (Chrzanowska-Wodnicka and Burridge, 1996; Pelham and Wang, 1997; Watanabe et al., 1999); mDia stimulates actin polymerization (Watanabe et al., 1999). The cell-strengthening response induced by force application to integrins, as well as focal adhesion assembly, has also been reported to be mediated by activation of the tyrosine phosphatase SHP2 which, in turn, activates the tyrosine kinase Src (Felsenfeld et al., 1999; von Wichert et al., 2003). In addition, mechanosensitive ion channels can be activated by force application to integrins (Glogauer et al., 1997; Munevar et al., 2004). The resultant influx of calcium into the cytoplasm can modulate contractility via calmodulin/caldesmon interactions and thus, actively influence both cytoskeletal organization (Sokabe et al., 1997) and cell-stiffening behavior (Helfman et al., 1999). Other studies suggest that the cell-strengthening response may be influenced by the level of resting tension (prestress) in the cytoskeleton prior to force application (Wang and Ingber, 1994; Pourati et al., 1998; Wang et al., 2002; Rosenblatt et al., 2004).

One potential limitation of these studies is that they focus on the effects of static mechanical stress. The reality is that cells commonly display both temporal and spatial variations in the level of stress they exert on their ECM adhesions (Beningo et al., 2001). The mechanical properties of cells also may differ depending on the frequency of force application (Fabry et al., 2001; Mack et al., 2004; Overby et al., 2005). Moreover, some cells exhibit distinct sensitivities to specific force frequencies (Goldschmidt et al., 2001; Hsieh and Turner, 2001; Coplen et al., 2003; Tanaka et al., 2003). Consistent with these observations, the morphology, composition, subcellular distribution, mobility and mechanical properties of integrin-based focal adhesions are also heterogeneous and dynamic (Smilenov et al., 1999; Katz et al., 2000; Zamir et al., 2000; Balaban et al., 2001). It is therefore crucial to elucidate how cells respond locally to dynamic as well as static mechanical cues in the ECM that are conveyed through integrin receptors. In particular, it is important to understand whether cells can mount multiple adaptive responses to different stress regimens, and if so, to determine which of these responses involve changes in the passive material properties of the cell, the level of pre-existing tension (prestress) in the cytoskeleton, active stress-induced molecular remodeling events, or any combination of these parameters.

In the present study, we therefore set out to examine how cells adapt to mechanical stress by stimulating cells with both static and dynamic force regimens. We accomplished this using a recently described magnetic-pulling cytometry technique (Matthews et al., 2004b). In this method, a permanent magnetic microneedle attached to a micromanipulator is used to apply sustained or pulsed tensional forces to magnetic microbeads bound to specific receptors on the surface membrane of cultured cells while simultaneously measuring nanometer-scale bead displacements using optical microscopy. These studies confirm that cells use multiple mechanisms involving different signaling pathways and different time scales to respond to stress sensed through integrin receptors.

Results

Immediate viscoelastic response of individual focal adhesions

To study how cells respond to static and dynamic mechanical forces, we applied controlled mechanical stresses to magnetic microbeads (4.5 μm diameter) coated with a synthetic RGD peptide that binds to integrin receptors on the surface membrane of cultured capillary endothelial cells (Fig. 1A) using permanent magnetic pulling cytometry (Matthews et al., 2004b). Past studies have shown that these RGD-beads ligate and activate integrin receptors, and induce focal adhesion formation at the site of bead binding in these cells (Plopper and Ingber, 1993; Meyer et al., 2000). To explore the earliest events involved in cellular adaptation to forces exerted on integrins, we applied brief (3 second) pulses of force (0 to 350 pN) to individual RGD-beads by controlling the distance of the needle tip to the bead with a micromanipulator. Resulting bead displacements were determined with sub-micrometer resolution using real-time optical microscopy in conjunction with computerized image analysis (Fig. 1B,C). The responses of individual beads to force applied in this manner have been shown to be due to the local mechanical properties of associated integrin-cytoskeleton linkages and recruited focal adhesion proteins (Matthews et al., 2004b).

When analyzed in this manner, typical viscoelastic-creep responses of individual focal adhesions (bead-integrin-cytoskeleton linkages) were observed, and the beads recoiled elastically to return almost completely to their starting positions when the force was removed (Fig. 1C). The elastic recoil of these beads has been previously shown to be approximately 0.5 seconds in these cells (Matthews et al.,

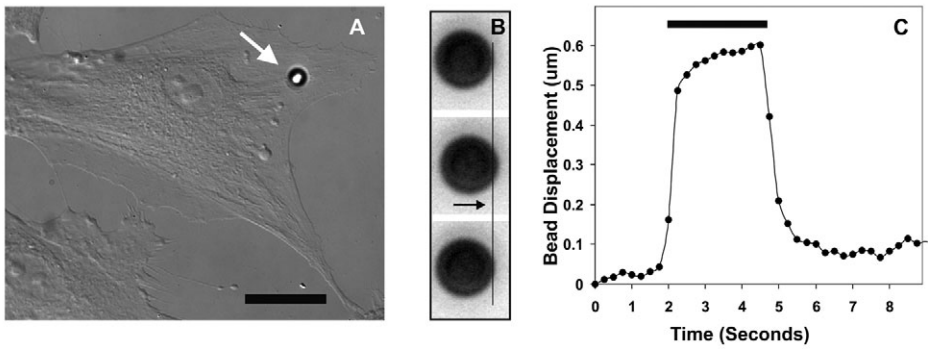

Fig. 1. Visualization and quantification of stress-induced displacements of integrin-bound magnetic microbeads on the surface membrane of cultured cells. (A) Differential interference contrast view of an adherent endothelial cell with an arrow indicating a single RGD-coated magnetic bead (4.5 μm diameter) bound to integrins on its apical surface. Bar, 20 μm. (B) A series of bright-field images recorded over 9 seconds showing bead position before (top), during (middle) and after (bottom) application of a 3-second force (130 pN) pulse (arrow indicates direction of force). (C) Bead displacement (μm) as a function of time before, during and after the 3-second force pulse (thick black line), as determined using computerized image analysis.

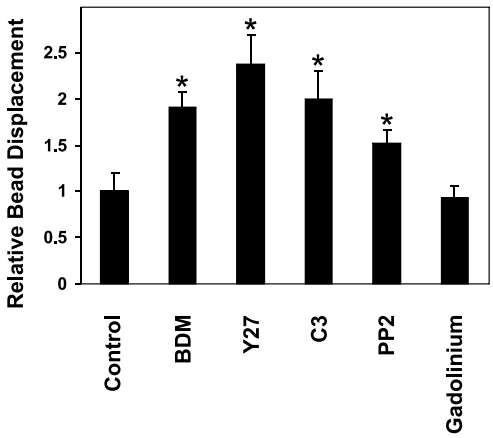

Fig. 2. Effects of modulators of cytoskeletal prestress and focal adhesion assembly on the immediate viscoelastic response of individual magnetic beads bound to surface integrin receptors. A 3-second force (130 or 350 pN) pulse induced less displacement when applied to RGD-beads bound to integrins on control cells compared with cells treated immediately prior to force application with BDM, Y27, C3 transferase, PP2 or gadolinium. Data represent the mean ± s.e.m.; **P*<0.05 compared with levels in the control.

2004b). Similar studies were then carried out in the presence or absence of the myosin ATPase inhibitor BDM, the ROCK inhibitor Y27632, or the specific Rho inhibitor, C3-transferase, to explore the roles of Rho and prestress (pre-existing isometric tension in the cytoskeleton) in this immediate viscoelastic response to mechanical stress applied through integrin receptors. Reduction of baseline cytoskeletal tension by these agents, which act by three different mechanisms, significantly decreased the stiffness of the bead-associated adhesions: bead displacements were approximately two times greater in treated versus control cells (Fig. 2).

To determine the possible involvement of tyrosine phosphorylation in the modulation of the physical properties of bead-integrin-cytoskeleton linkages, and hence in the immediate viscoelastic response, we carried out similar studies in the presence of PP2, an inhibitor of Src-family tyrosine kinases (Hanke et al., 1996) which has been shown to modulate both focal adhesion formation (Miyamoto et al., 1995a; Miyamoto et al., 1995b) and the ability of cells to adapt to mechanical force (Felsenfeld et al., 1999; von Wichert et al., 2003). We found that PP2 treatment also inhibited the cell-stiffening response, as bead displacements were significantly greater in the presence of PP2 compared with the control (Fig. 2).

Past studies suggested that mechanical force application to integrins can activate mechanosensitive ion channels and trigger calcium entry into cells which may influence cell mechanics by modulating cytoskeletal structure or contractility (Glogauer et al., 1997; Glogauer et al., 1998; Munevar et al., 2004). To explore this possibility in endothelial cells, we applied a range of forces (150 pN to 5 nN) to cells via attached RGD-beads using ceramic (Matthews et al., 2004b) or electromagnetic (Matthews et al., 2004a; Overby et al., 2005) needles while simultaneously measuring changes in $[Ca^{2+}]_i$ using ratio-imaging in cells labeled with Fura2-AM. Application of high (1 to 5 nN) levels of mechanical stress to integrin-bound beads resulted in induction of calcium entry within 2 to 5 seconds after force application which returned to baseline within 30 to 90 seconds (Fig. 3). Moreover, this calcium-signaling response was completely blocked (Fig. 3C) when these bovine endothelial cells were treated with the stretch-sensitive ion-channel blocker, gadolinium chloride (Yang and Sachs, 1989), at the same dose (25 μM) that has been shown to block stretch-sensitive ion-channel function in human endothelial cells (Naruse and Sokabe, 1993; Naruse et al., 1998). However, when we applied lower levels of stress (<1 nN), similar to those that we used to analyze the immediate viscoelastic response, no significant changes in $[Ca^{2+}]_i$ could be detected. Consistent with this finding, treatment of cells with gadolinium chloride had no effect on this immediate stiffening behavior (Fig. 2).

Thus, the immediate cellular response to a 3-second force pulse is highly sensitive to the level of tensile prestress in the cytoskeleton, and to tyrosine kinases that mediate focal adhesion formation, but apparently not to calcium signaling through mechanosensitive ion channels.

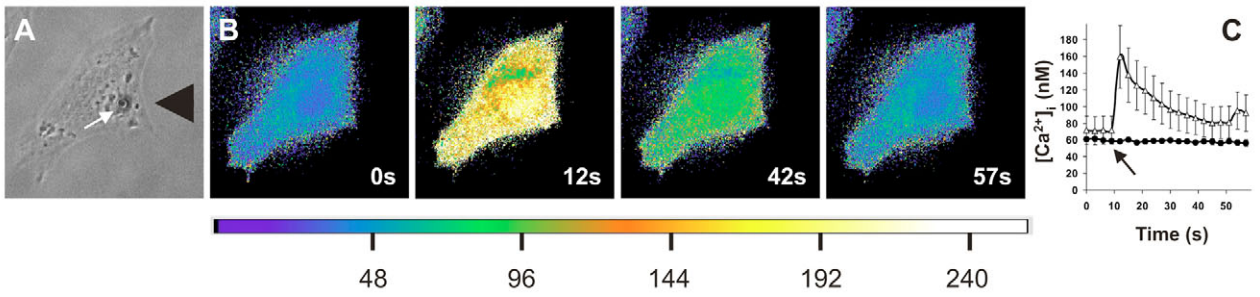

Fig. 3. Application of high (nN) levels of stress to cell-surface integrins increases intracellular calcium. (A) Phase-contrast view of an adherent endothelial cell with attached RGD-bead (white arrow) bound to integrins on the apical cell surface. Black arrowhead indicates position of the tip of the electromagnet. (B) A time series of pseudocolored fluorescence images of the cell shown in A after mechanical stress (5 nN) was applied with the magnet. These pseudocolored images demonstrate a transient stress-induced increase in $[Ca^{2+}]_i$ as a brief shift in color from blue to yellow, as detected using Fura-2AM ratio-imaging (color bar indicates $[Ca^{2+}]_i$ in nM). The times after start of the time-lapse series are indicated in seconds; the force pulse was applied at 9 seconds in this series. (C) Plot of average $[Ca^{2+}]_i$ for control (◇) and gadolinium chloride-treated (●) cells as a function of time; the inhibition of stress-induced calcium influx by gadolinium was statistically significant (*P*<0.002; error bars indicate s.e.m.). Black arrow indicates when the 3-second force pulse was applied.

Early adaptive strengthening in response to pulsatile forces

A rapid form of force-dependent strengthening of integrin-cytoskeleton linkages has been observed when a static force is applied to smaller (1 μm diameter) beads coated with integrin ligands for more than approximately 10 seconds using an optical laser trap in fibroblasts (Choquet et al., 1997; Felsenfeld et al., 1999; Giannone et al., 2003). In those studies, adaptive strengthening was defined by the cell's ability to progressively strengthen its adhesions over time and thereby, physically pull the bead out of the laser trap. To explore whether endothelial cells exhibit similar adaptive strengthening in response to a rapid-force pulse, we applied 3-second force pulses similar to those described in Fig. 1 to the same RGD-bead every 5 minutes over a 20 minute period. These studies revealed that there was no difference in bead displacement between that induced by first versus subsequent force pulses (data not shown). Thus, the 3-second mechanical force pulse we applied did not result in a long-standing change in the integrin-cytoskeleton linkage within the bead-associated focal adhesions or cell mechanical responsiveness.

We then applied a dynamic force regimen composed of multiple consecutive 3-second force pulses (130 pN) with 4-second relaxation intervals (Fig. 4A) to observe whether cells could mount a cumulative time-dependent response to a pulsatile force regimen. The relaxation time was chosen based on the known elastic recoil time (0.5 seconds) of bound RGD-beads in these cells (Matthews et al., 2004b) to ensure that the beads would recoil fully prior to stimulation with the next force pulse. In these experiments, we observed a small but significant pulse-to-pulse attenuation in bead displacement that saturated by the third pulse (Fig. 4B,C). This response was suppressed by treating cells with BDM or Y27632 to inhibit cytoskeletal tension generation, or by cooling the cells to 4°C in order to slow biochemical remodeling (Fig. 4C), whereas once again, treatment of cells with gadolinium chloride had no effect (data not shown). Thus, this adaptive strengthening behavior appears to require changes in molecular assembly and maintenance of cytoskeletal tension, but not stress-dependent ion signaling.

Later adaptation in response to sustained stresses

We next explored how the duration of force exposure influences the cell's ability to mount an adaptive strengthening response. To determine the minimal time an applied force must be prolonged in order for the cell to develop a stable change in adhesion mechanics, we probed cells bound to RGD-beads with a 3-second force pulse (250 pN) before and after exposing the same bead to a similar level of force that was prolonged for increasing periods of time (5 seconds to 5 minutes) (Fig. 5A). These studies revealed that the cellular response to the second brief force pulse was significantly attenuated (i.e. cells appeared stiffer) if the cells were exposed to an intervening sustained force of 15 seconds or longer (Fig. 5B). Interestingly, although treatment of cells with BDM, Y27632, C3 or PP2, decreased the stiffness of integrin-associated focal adhesions when probed by a single 3-second force pulse (Fig. 2), cells were still able to mount this slower strengthening response to a sustained (2 minute) stress when cytoskeletal tension generation, tyrosine kinase activity, or stretch-sensitive ion channels were separately inhibited (Fig. 5C). By contrast, this strengthening response was completely inhibited when cells were chilled to 4°C (Fig. 5C). Thus, this slow adaptive strengthening behavior induced by exposure to forces sustained for more than 15 seconds appears to be mechanistically distinct from the response induced by multiple short (3-second) pulses separated by short (4-second) relaxation intervals (Fig. 4).

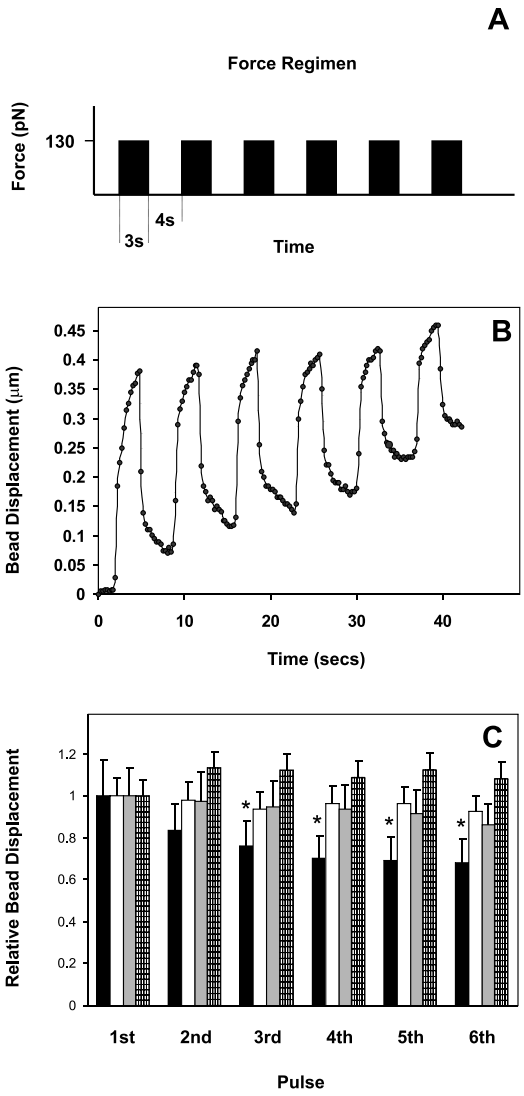

Fig. 4. Early cellular strengthening response to oscillatory force. (A) Schematic of experimental design. Six consecutive 3-second force pulses (130 pN) were applied to bound RGD-beads, separated by brief (4-second) intervals. (B) Representative example of bead displacements measured in response to the pulsatile force regimen shown in A. Note strengthening of adhesions following pulses 2 and 3, as indicated by decreased displacements. (C) Average relative RGD-bead displacements induced by the pulsatile force regimen. Black bars, control; white bars, BDM; gray bars, Y27632; hatched bars, 4°C; **P*<0.05 where indicated compared with the level in the first pulse respective experimental condition; note that only controls exhibited significant attenuation.

Repositioning responses induced by prolonged force application

To explore in greater depth how cells respond to sustained physical stresses analogous to those experienced during cell spreading and motility, we monitored the time-dependent

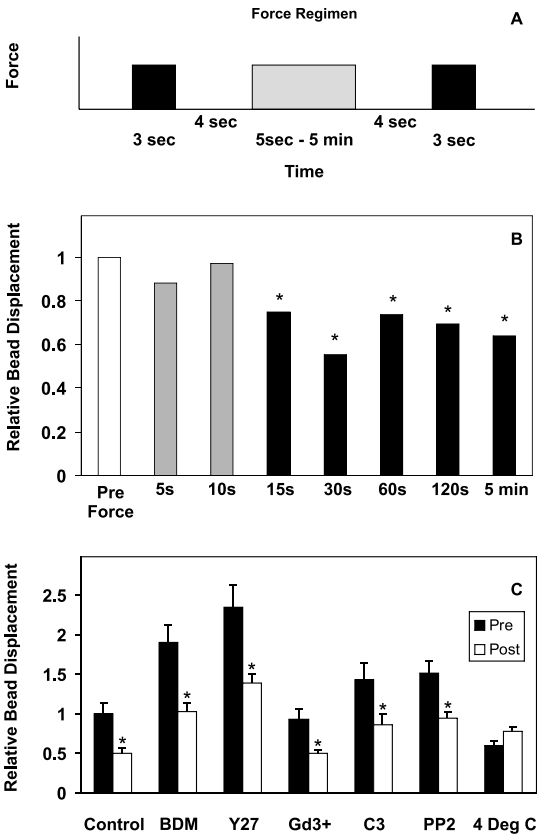

Fig. 5. Adaptive strengthening of bead-integrin-cytoskeleton linkages induced by prolonged static force. (A) Schematic of experimental design. Brief (3 second) force pulses (black bars) were applied to surface-bound RGD-beads before and after static forces that were sustained for different times (grey bar) in order to determine if prolonged forces induce stable cell strengthening. (B) Prolonged force (250 pN) of ≥15 seconds duration induced strengthening of RGD-bead adhesions. Relative bead displacements represent displacements produced by the second 3-second force pulse divided by that induced by the first force pulse (white bar) (**P*<0.05; grey bars indicate no significant difference compared with pre-force levels). (C) Strengthening of RGD-bead adhesions in response to application of force (130 or 350 pN) prolonged for 2 minutes in the absence (black bars) or presence (white bars) of BDM, Y27632, gadolinium, C3 transferase, PP2 or exposure to 4°C (**P*<0.05 compared with pre-force levels).

changes in the position of the integrin-bound beads on the endothelial surface in response to prolonged magnetic stress. Without force application, adherent magnetic RGD beads spontaneously translocated over the surface of the cell towards the nucleus at a rate of approximately 65 nm/minute regardless of their starting position on the cell (Fig. 6A). Similar translocation behavior has been observed previously using non-magnetic microbeads bound to integrin receptors (Choquet et al., 1997).

To explore the effects of force application on integrin translocation, we applied magnetic forces to beads oriented between the nucleus and the tip of the magnetic microneedle so that tensional forces were applied centripetally, and hence in a direction opposite to that of normal bead movement under baseline conditions. When a low level of magnetic force (130 pN) was applied to surface-bound RGD-beads and maintained for an extended time (1 to 2 minutes), the beads were initially displaced toward the magnet and away from the nucleus; however, a significant fraction of the beads eventually stalled and then reversed their direction (Fig. 6A,B). These beads moved against the magnet's force gradient and towards the nucleus, with some beads actually ending up closer to the nucleus than they were at the time the force was first applied (Fig. 6B). On average, a magnetic force of 130 pN was able to drag the RGD-beads approximately 50 nm away from the nucleus within 10 to 20 seconds after force application, but then the beads were retracted in the opposite direction to the magnetic force and were about 40 nm closer to the nucleus relative to their starting position after 2 minutes (Fig. 6A). Of the beads that the cell was able to move against the force gradient, roughly 50% did so within 20 seconds of force application, and more than 95% reversed direction by 90 seconds.

By contrast, when higher levels of force (350 pN) were

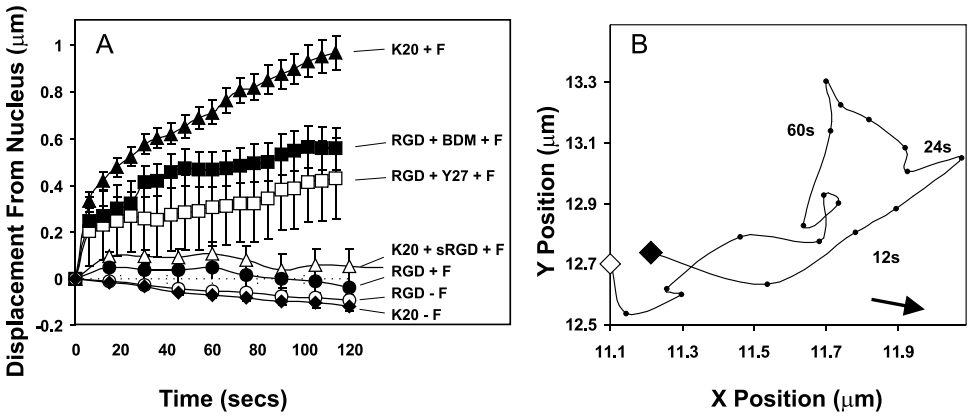

Fig. 6. Large-scale translocation of bead adhesions in response to prolonged force. The position of individual surface-bound magnetic beads coated with RGD peptide or K20 antibody was recorded in the absence or presence of sustained (2 minutes) application of force (130 pN) in a centripetal direction, opposite to that in which the beads normally move on the surface of the cell. (A) Measured displacements of RGD-beads in the absence of force (○; –F) and in the presence of force (+ F) without (●) or with Y27 (□) or BDM (■). Displacements of K20-beads in the absence of force (◆; –F) or presence of force (+F) without (▲) or with soluble RGD peptide (sRGD, △). (B) Representative *X-Y* plot of positions of a representative RGD-bead before (◆), during (black line), and after (◇) 2 minute application of force (130 pN). The bead was initially displaced toward the magnet, but was then actively retracted against the magnetic gradient and towards the nucleus during the course of force application (arrow indicates direction of applied force).

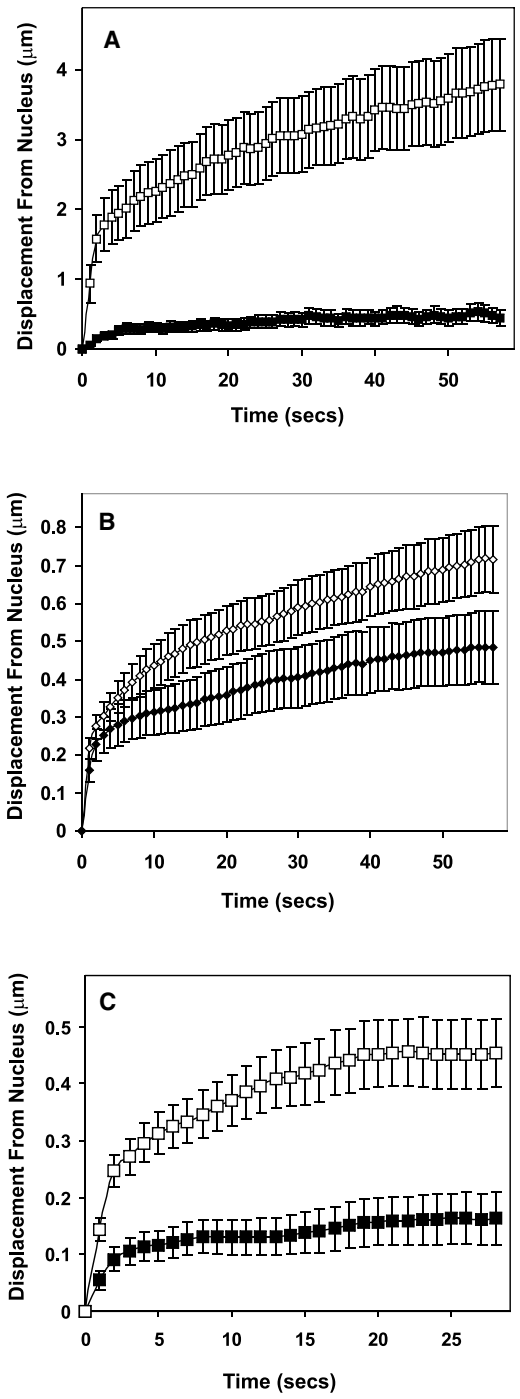

Fig. 7. Contributions of Rho, mechanosensitive ion channels and Src tyrosine kinases to the adaptive repositioning response to prolonged stress. RGD-beads bound for 10 minutes to cells were exposed to a prolonged (60 seconds in A,B; 30 seconds in C) stress (350 pN) applied in a centripetal direction opposite to that which beads normally move on the cell surface. (A) Bead displacements relative to the nucleus were measured in cells exposed to the BioPORTER proteofection reagent in the absence (●) or presence (□) of C3 transferase. (B) Bead displacements measured in the absence (◆) or presence (◇) of gadolinium chloride. (C) Bead displacements measured in the absence (■) or presence (□) of PP2. Error bars indicate s.e.m.; $P<0.05$ at times greater than 30 seconds compared with controls in all graphs.

applied to the same beads, cells were not able to overcome or balance the applied stress, and the average bead displacement away from the nucleus increased progressively over time (Fig. 7A). Nevertheless, the cells were able to maintain adhesion to the beads, and the maximal bead displacement was limited to less than 500 nm, even after 1 minute of prolonged force application. These data confirm that cells can sense local mechanical stresses applied to integrins, and that they respond by actively generating large traction forces, somewhere between 130 and 350 pN, to counter and overcome these stresses.

To determine the requirement of integrin activation for this repositioning response, we applied magnetic forces (130 pN) to beads coated with a non-activating anti-β1 integrin antibody (K20) that binds integrins but does not activate the receptors or induce focal adhesion formation (Miyamato et al., 1995a,b; Miyamato et al., 1995b). The K20-beads behaved similarly to RGD-beads in the absence of applied stress, and moved towards the nucleus at similar rate (Fig. 6A). By contrast, when magnetic stress was applied, virtually all of the K20-beads (19 of 20) moved passively in the direction of applied force, and they failed to adapt by reversing their direction (Fig. 6A). These beads displaced more than twice as far (>1000 nm) as control beads when forces were prolonged for up to 2 minutes. Importantly, when soluble RGD ligand was added to cells already bound to K20-beads to ligate the binding sites of the antibody-bound integrins and thereby chemically activate these receptors, the cells regained the ability to pull the K20-beads against the magnetic force and towards the nucleus, hence mimicking the behavior exhibited by integrin-activating RGD-beads (Fig. 6A). Thus, integrin activation is required for this large-scale strengthening and repositioning response. Prolonged magnetic forces were then applied to cells that were pre-treated with either BDM or Y27632 to determine whether myosin motors contribute to this response. Dissipation of cytoskeletal tension inhibited the cell's ability to adaptively reposition the beads and pull them against the applied magnetic force gradient (Fig. 6A).

To directly explore whether the small GTPase Rho contributes to this large-scale repositioning response, we proteofected cells with C3-transferase and applied the higher force level (350 pN) to cell-surface integrin-bound RGD-beads to maximally stress the focal adhesions without dislodging the beads from the cells. Bead displacement increased over sevenfold (>3500 nm displacement) in response to force when cells were treated with C3, and the displacement response did not saturate even after 1 minute of continuous force application (Fig. 7A). Thus, cells require active Rho to produce chemical signaling responses and generate traction forces that produce large-scale bead retraction in response to prolonged mechanical stresses.

Tyrosine kinases also have been shown to mediate maturation of focal adhesions (Miyamoto et al., 1995a; Miyamoto et al., 1995b) as well as force-induced adhesion growth and strengthening (Felsenfeld et al., 1999). When cells were mechanically stressed through bound RGD-beads in the presence of PP2 to inhibit Src tyrosine kinases, cells were less able to resist the applied stress and move the beads against the force gradient and towards the nucleus (Fig. 7C). Interestingly, although we could not detect a change in cytosolic calcium concentration when cells were exposed to prolonged

application of this level of force (350 pN for 90 seconds), treatment of cells with gadolinium chloride inhibited their ability to resist the applied stress and pull the beads towards the nucleus (Fig. 7B). These experiments suggest that Src kinases and mechanical force-induced activation of stretch-sensitive ion channels may contribute to this repositioning response to prolonged force application, even though these same signaling molecules did not appear to be contribute to the earlier adaptive response to stress (Fig. 4C).

Discussion

To better understand how cells adapt to changes in mechanical forces exerted on their ECM adhesions or to changes in ECM rigidity, we used magnetic-pulling cytometry (Matthews et al., 2004b) to apply static or pulsatile tensional forces to cells via ligand-coated magnetic microbeads bound to integrin receptors on the surface membrane of cultured bovine capillary endothelial cells. Analysis of force-induced bead displacements revealed that when integrins are stressed, local integrin-cytoskeleton linkages within bead-associated focal adhesions (Plopper and Ingber, 1993; Matthews et al., 2004b) exhibit an immediate viscoelastic response, as well as at least three distinct forms of strengthening behavior that act over different time scales.

The first type of adaptive response was an early strengthening in response to application of three or more closely spaced short pulses that occurred over the time scale of seconds. This early strengthening response can be suppressed by either disrupting myosin-dependent contractility using Y27632 or BDM, or by slowing overall biochemical activity by cooling the cell to 4°C. The second more robust strengthening response occurred after 15 seconds of prolonged force and was unaffected by inhibitors of myosin contractility, stretch-activated channels or the tyrosine kinase Src, but was sensitive to a reduction in temperature. The third type of strengthening behavior occurred in response to prolonged application of stress for over 60 seconds and was characterized by retraction of bound beads in the opposite direction to applied magnetic force. Force application to integrins alone was not sufficient to induce these adaptation responses; the integrins also had to be chemically activated through occupancy of the RGD ligand-binding site. Importantly, these different strengthening behaviors also were distinct in that they exhibited different sensitivities to drugs that dissipate cytoskeletal prestress, inhibit Rho signaling, or interfere with stretch-activated ion channel function. These findings suggest that the cellular response to mechanical force occurs over multiple time scales and involves diverse biochemical mechanisms.

The magnetic-pulling cytometry method used in the present study offers a number of advantages over past techniques used to explore adaptive cellular strengthening behavior. Most importantly, compared with an optical laser trap which may be used to only briefly trap integrin-bound beads on the surface of cells (Choquet et al., 1997), the magnetic needle can apply forces to similarly bound beads for much longer periods of time. This is because the laser trap generates a very steep force gradient at its edges, and when a cell adaptively reinforces its adhesions to the bead and moves it to the perimeter of the trap, the resistance force drops rapidly, allowing the bead to escape. Once removed, the bead is no longer stressed, and the cell readjusts itself to a no-force state (Choquet et al., 1997). Thus, the optical laser method is limited to a 10-15 second window in which to explore the mechanisms that regulate force-induced cellular strengthening behavior. By contrast, using the magnetic needle, which generates a broad magnetic field gradient near the needle tip, forces can be applied to beads for longer periods of time (minutes to days), and mechanical force can be maintained on the bead during and after adaptive cellular strengthening has occurred. In addition, the levels of force that can be applied using the optical trap, which vary for different-sized beads, are limited because of the risk of thermal injury to the cells by the laser at higher energy levels. The magnetic needle, on the other hand, is non-toxic to the cell even at close proximity, and high levels of force (up to 10 nN on 4.5 μm beads) may be safely applied. Finally, unlike the optical trap, the magnetic needle can apply pulses of force to beads to induce bead displacements that can be used to estimate the local mechanical properties of the cell, and in particular, of bead-associated integrin-cytoskeleton linkages. Using this method, we quantitatively determined that cells exert a sustained force of between 130 pN and 350 pN on a single 4.5 μm RGD-bead attachment.

By taking advantage of this last feature of the magnetic method and estimating local cellular mechanical properties before and after brief or sustained force application, we were able to demonstrate stress-induced stiffening of integrin-cytoskeleton linkages within bead-associated focal adhesions. Because the elastic time constant of the viscoelastic response estimated using the viscoelastic-creep model of Bausch et al. (Bausch et al., 1998) of these beads in these same cells is approximately 0.5 seconds (Matthews et al., 2004b), the 4 second lag period between force pulses in all our experiments provides approximately a tenfold longer period for bead recoil to reach equilibrium after force release, prior to the next force pulse. This is important because an inability of the integrin-cytoskeleton linkages to fully recoil prior to the next pulse could result in decreased bead displacement and be misinterpreted as cellular strengthening. In fact, RGD-beads do not return to their exact start position following 3-second force pulses (Matthews et al., 2004b); this leads to an apparent shift in the baseline position of the beads following each successive pulse when applied one after the other. However, because stiffening responses are abolished when the cells are either chilled or treated with drugs without altering their passive viscoelastic properties, the observed reduction in bead displacements following force application is more likely to be the result of active cellular adaptation, rather than incomplete relaxation of the integrin-cytoskeleton linkages or changes in their passive mechanical properties.

In designing these adaptation experiments, the period between any two pulses must be long enough to allow adequate time for complete elastic recoil of the integrin-cytoskeleton linkages. However, it also must be short enough to prevent cellular readaptation to no-force conditions when force is released because the adaptive reinforcement response to stresses applied to integrins has been shown to be lost within seconds following removal of mechanical stress (Choquet et al., 1997). Focal adhesions also decrease in size within seconds after dissolution of cytoskeletal prestress using chemical mediators (Balaban et al., 2001). RGD-beads induce focal adhesion formation at the bead-binding site in the bovine

capillary endothelial cells we used here (Plopper and Ingber, 1993; Wang et al., 1993; Matthews et al., 2004b), which contribute significantly to the local mechanical properties of the bead-integrin-linkages (Matthews et al., 2004b). The reduction in bead displacement observed upon repeated force application may therefore be due to stress-induced restructuring of focal adhesion proteins that link these beads to the cytoskeleton (Giannone et al., 2003). Alternatively, cellular preconditioning prior to reaching an equilibrium state (analogous to preconditioned pseudoelastic responses seen in tissues) (Fung, 1993) may also in part explain the attenuation in force-induced bead displacement we observed.

The earliest adaptive strengthening response that we observed was detected using a pulsatile force regimen composed of multiple brief (3-second) force pulses, separated by short (4-second) relaxation periods: the displacements of the second and third pulses were attenuated relative to the first. This early adaptation behavior was prevented by dissipating cytoskeletal prestress with inhibitors of either myosin ATPase or ROCK, or by cooling the cells to 4°C to suppress biochemical remodeling events. These data suggest that the cell adjusts both the level of tension it generates in its cytoskeleton and the molecular composition of its focal adhesion in response to repeated forces, and that this remodeling occurs over the time scale of seconds. Similar pulsed experiments using magnetic beads on fibroblasts, however, did not find a stiffening response (Bausch et al., 2001). Several methodological differences may explain this discrepancy, including differences in the duration of the force pulse (2 versus 3 seconds), the cell types (fibroblasts versus capillary cells), and other experimental conditions (e.g. bead-coating procedures) that could affect bead binding or the quality of associated integrin-cytoskeleton linkages. Given that different cells may respond differently to force depending on the frequency of force application (Goldschmidt et al., 2001; Hsieh and Turner, 2001; Coplen et al., 2003; Tanaka et al., 2003; Mack et al., 2004), and that the propagation of force through the cell varies depending on the level of prestress in the cytoskeleton (Hu et al., 2005), the difference between our results also may relate to the different frequencies of force application we used, or to differences in cytoskeletal prestress in the cells we studied.

When localized force is applied to single integrin-bound beads on the surface of cells, the strengthening response occurs only in the stressed bead, and not in neighboring beads (Choquet et al., 1997). Cells also can vary the level of traction force they exert on individual focal adhesions (Tan et al., 2003). Our results relating to the early tension-dependent strengthening response are consistent with these findings and suggest that cells respond locally to mechanical stimulation by augmenting the level of tension preferentially in the vicinity of the applied force. Given that stress application to integrins promotes focal adhesion assembly (Riveline et al., 2001), this local increase in cytoskeletal tension may feed back positively to promote biochemical assembly events that, in turn, lead to localized strengthening of individual adhesions and altered sensitivity to later waves of force application. This enhanced contractility and cytoskeletal restructuring in response to stress is also similar to how increased ECM stiffness and cell-shape distortion increase cell contractility and enhance the ‘responsiveness’ of cells to vasoactive agents (Polte et al., 2004), as well as to growth factors during tumor progression (Paszek et al., 2005).

The second type of strengthening behavior that we identified was a later adaptive response that was observed only after cells were continuously exposed to force for at least 15 seconds. This form of adaptive strengthening was distinct from the earlier response in that it was not sensitive to inhibition of cytoskeletal tension generation, Rho, Src tyrosine kinase or stretch-sensitive ion channel activity. Force application to cell-ECM adhesions has been shown to induce localized focal adhesion growth by activating ROCK and mDia (Riveline et al., 2001). Thus, our finding that cells continued to exhibit this late adaptive strengthening even when Rho and cytoskeletal tension generation were inhibited suggests that some other type of biochemical remodeling mechanism must exist to explain the increased adhesion stiffness in response to stress that we observed. Several explanations for these findings are possible, including mechanisms of focal-adhesion assembly that are independent of Rho, as well as other forms of biochemical modification of the surrounding cytoskeleton or membrane that could provide increased mechanical stability. For example, Rac-dependent formation of focal complexes is independent of Rho (Nobes and Hall, 1995; Galbraith et al., 2002). Rho inhibition also does not prevent incorporation of α-actinin into focal adhesions which can link integrins to the actin cytoskeleton (Pavalko et al., 1991; Geiger et al., 2001; von Wichert et al., 2003), and significantly alter the mechanics of actin gels (Wachsstock et al., 1993). In addition, physical forces can alter phosphorylation of focal adhesion proteins in detergent-extracted cytoskeletons of adherent cells (Tamada et al., 2004): this is another way in which mechanical stress may directly alter the mechanical properties of focal adhesions.

Finally, in addition to strengthening their adhesions when mechanically stressed, the cells also responded to applied forces by physically retracting integrin-bound beads in a direction opposite to that of the applied stress (i.e. towards the nucleus). This behavior has been observed in many cell types with many different types of beads coated with integrin ligands (Choquet et al., 1997; Felsenfeld et al., 1999; Giannone et al., 2003). In these past studies, it was not possible to separate retraction of beads from local strengthening of bead adhesions because the laser trap can only hold the bead in one position; it cannot measure the mechanical properties of the bead adhesions. By contrast, using the magnetic technique, we were able to separate these two responses. We found that retraction of engaged adhesions required integrin activation, Rho signaling and cytoskeletal tension generation, as well as activation of both Src and mechanosensitive ion channels. By contrast, force-induced stiffening of bead adhesions was maintained in the presence of all of these inhibitors (Fig. 5C). How cells regulate the movement of strengthened adhesions in response to sustained versus repetitive forces is a question that warrants further investigation in the future.

Although we did not observe global changes in intracellular calcium concentration in cells exposed to prolonged application of the relatively low (150 to 350 pN) levels of force used to analyze cell mechanical behavior, we did find that gadolinium chloride inhibited the cells ability to retract beads against a force gradient in response to prolonged (>60-second) stress application. Gadolinium has been shown to block stretch-sensitive calcium channels in many cells (Naruse and

Sokabe, 1993; Naruse et al., 1998); however, it is not selective as it also blocks L-type calcium channels as well as delayed rectifier potassium channels (Hongo et al., 1997). Nevertheless, our finding that gadolinium blocks stretch-activated calcium signaling when higher stress levels (1-5 nN) were applied to integrins in these cells indicates that the bovine capillary endothelial cells we studied here have stretch-sensitive calcium channels that can be inhibited by gadolinium. These stretch-sensitive channels are not required for the early adaptation responses, whereas some type of gadolinium-sensitive channel is required for the cell's repositioning response to prolonged stress. Although we could not detect force-activated increases in cytosolic calcium under conditions of prolonged application of low to moderate levels of stress (350 pN) that induced this repositioning response, it is still possible that low levels of calcium that are below the resolution of our calcium-imaging system may enter cells via stretch-activated channels. These low ion fluxes may not be large enough to trigger calcium-induced release of intracellular stores of calcium that are normally required for full-fledged calcium spikes (Lee et al., 1999), as we observed in cells exposed to high (nN) stress (Fig. 3C). But if sustained over time, low levels of calcium influx at the bead binding site could influence local interactions between integrins, focal adhesion proteins, and the cytoskeleton and thereby alter bead translocation across the membrane. Alternatively, the effects of gadolinium on other targets (e.g. delayed rectifier K^+ channels) could contribute to this response. Interestingly, treatment of fibroblasts with gadolinium chloride inhibits cell migration and decreases traction forces in the front but not at the rear of the cell (Munevar et al., 2004). Our finding that gadolinium inhibits large-scale movement of RGD-beads, but not the immediate viscoelastic response to force, is consistent with this idea that stretch-sensitive ion channels contribute to force generation and movement of certain focal adhesions, whereas it does not influence others that may experience different micromechanical cues.

The levels of force (150 to 350 pN) applied to individual bead-associated focal adhesions in the present study are probably physiologically relevant as they are similar to the levels of stress cultured cells exert on individual substrate-associated focal adhesions (Balaban et al., 2001; Tan et al., 2003). Thus, our finding that cells have the ability to distinguish between mechanical forces applied over different time scales, and to separately regulate the mobility and the mechanical properties of stressed integrin-cytoskeleton linkages, has several important implications. The ability to distinguish mechanical signals prolonged over a period of minutes from forces exerted over seconds may be important for the cell's ability to exhibit robust directional movement in response to a sustained stress in an environment that may experience multiple mechanical perturbations on a shorter time scale. By contrast, the stiffening response produced by rapid force pulses may serve to protect the cell against physical and biochemical injury, as previously described in other cells (Glogauer et al., 1998; Kainulainen et al., 2002). In fact, we have observed that beads bound to transmembrane scavenger (acetylated-LDL) receptors that do not efficiently couple to the cytoskeleton and hence, do not exhibit these strengthening responses, are torn out of the membrane in our endothelial cells when similar forces (350 pN) are applied. Additionally, our cells respond to prolonged force application by stiffening their integrin-cytoskeleton linkages to balance the applied stress, and by exerting actomyosin-based tensional forces that physically pull the beads toward the cell center. The relative contribution of local versus more distal responses ultimately determines whether the stressed integrin-cytoskeleton linkages will be repositioned or strengthened through increased focal adhesion assembly locally, and this ultimately influences the mobility of the whole cell.

In summary, we used a magnetic pulling cytometry technique to measure the mechanical properties of individual magnetic microbead-associated focal adhesions (integrin-cytoskeleton linkages) in order to study how cells adaptively strengthen in response to static and dynamic mechanical force regimens. Three mechanistically and temporally distinct adaptive strengthening behaviors were identified, two of which involve stiffening of adhesion structures (an early and a later form), and a third response that involves the large-scale repositioning and retraction of the stressed adhesions. Our data add additional support for the concept that individual integrin-containing focal adhesions function as local mechanosensors (Wang et al., 1993; Ingber, 1997; Geiger and Bershadsky, 2001). They also show that the adaptive strengthening behaviors exhibited by these adhesions are not mutually dependent: adhesion stiffening and remodeling can occur in the absence of repositioning and retraction. Understanding the molecular and biophysical mechanisms by which cells locally regulate these complex mechanical responses may clarify how cells change shape and control their migratory behavior.

Materials and Methods

Experimental system

Bovine capillary endothelial cells (passage 10 to 15) were maintained at 37°C in 10% CO_2 on tissue culture dishes in low glucose Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Hyclone), 10 mM HEPES (JRH-Biosciences) and L-glutamine (0.292 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) (GPS), as previously described (Chen et al., 1997; Yu et al., 2001). In preparation for experiments, cells were cultured in DMEM with 0.5% FCS for 24 hours, trypsinized (Trypsin-EDTA, Gibco), and then plated (1.5×10^4 cells/dish) onto glass-bottomed 35 mm dishes (MatTek Corp) that were pre-coated with 500 ng/cm^2 fibronectin (Becton Dickinson). Cells were maintained in the MatTek dishes for 18-24 hours prior to the experiment in serum-free DMEM with 10 mM HEPES, 0.5% bovine serum albumin (BSA), 10 μg/ml human high-density lipoprotein (Intracell), 5 mg/ml holo-transferrin (Collaborative Research) and GPS.

Tosyl-activated super-paramagnetic microbeads (4.5 μm diameter; M-450 Dynabeads, Dynal Biotech, WI) were coated with either synthetic RGD-containing peptide (peptite-2000, Integra) or non-integrin activating anti-β1 antibody (K20, Immunogen) in pH 9.4 carbonate buffer as previously described (Plopper and Ingber, 1993; Wang et al., 1993). Immediately before an experiment, adherent cells were incubated with beads (~20 beads/cell) for 10 minutes and then washed multiple times with PBS to remove unbound beads prior to magnetic stress application. To maintain proper pH throughout the experiment, these studies were carried out in bicarbonate-free medium consisting of Hanks balanced salts (Sigma), 10 ml/l MEM non-essential amino acids (Sigma), 20 ml/l MEM essential amino acids (Sigma), 2 mM L-glutamine (Sigma), 10 mM HEPES pH 7.3 and 1% BSA.

In experiments analyzing the role of cytoskeletal prestress, cells were treated with 2,3-butanedione 2-monoxime (BDM; 10 mM) or Y27632 (Y27; 20 μM) 10 minutes after beads were added to cells, immediately prior to force application. In experiments analyzing the role of the Src family of protein tyrosine kinases, cells were treated with 25 μM PP2 [4-amino-5-(4-chloro-phenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine; EMD Biosciences, LaJolla, CA] for 30 minutes prior to force application. Gadolinium chloride (Sigma; St Louis, MO) was used at a concentration of 25 μM to inhibit stretch-sensitive ion channel function (Naruse and Sokabe, 1993; Naruse et al., 1998). BioPORTER protein transfection reagent (Gene Therapy Systems, CA) was used to introduce recombinant C3-transferase protein (Cytoskeleton, Denver, CO) into the cytoplasm of living endothelial cells, as previously described (Mammoto et al., 2004). In brief, C3-exoenzyme (4 μg) was added to the dried BioPORTER along with 100 μl of PBS, incubated for 5 minutes at room temperature, vortexed gently for 3 to 5 seconds at low speed, and added to 1 ml of DMEM. For experiments using C3 proteofection, cells plated for 24 hours

on MatTek Petri dishes were incubated in C3/BioPorter solution for 4 hours, washed three times with PBS, and then cultured for 18 hours in DMEM containing 10% serum prior to experimental analysis. In control studies, the BioPORTER reagent was added alone. Experiments carried out in parallel confirmed that the BioPORTER reagent efficiently delivered the Rho-inhibiting toxin, C3 exoenzyme, into the cytoplasm of approximately 90% of bovine capillary endothelial cells, as indicated by disruption of actin stress fibers and disassembly of focal adhesions when analyzed by fluorescence microscopy. Similar effects have been previously demonstrated by microinjecting C3 exoenzyme into individual cells (Ridley and Hall, 1992; Ridley et al., 1992; Hall, 1998; Etienne-Manneville and Hall, 2002; Mammoto et al., 2004).

Magnetic-pulling cytometry

The permanent magnetic microneedle system we used to apply forces to magnetic beads has been previously described (Matthews et al., 2004b). Briefly, the system consists of a standard stainless steel needle attached to a permanent neodymium iron boron disc magnet (Edmund Industrial Optics, NJ) attached to an aluminum rod that is mounted on a microscope micromanipulator (Eppendorf, Germany). Calibration of forces generated by the magnetic needles was carried out by pulling the 4.5 μm Dynal magnetic microbeads through glycerol solutions of known viscosity, as described (Alenghat et al., 2000). To measure the local mechanical properties of cells with bound beads cells were maintained at 37°C using a heated stage (Omega Engineering Inc., CT) and visualized on a Nikon Diaphot 300 microscope (Nikon, Japan). The magnetic needle was then used to apply force pulses to the bead while bead displacement was measured optically. The manipulator speed was set to 1000 μm/second, and the tip was oriented at 45° relative to the substrate, and positioned within the culture medium 600 μm away from the cell-bound beads to be tested. The needle tip was rapidly moved within 70 to 125 μm from the bead, held in position for between 3 seconds to 5 minutes, and then quickly returned to its original position using the micromanipulator. Time-lapse imaging (4 Hz) with a CCD camera (Hamamatsu, Japan) attached to the microscope was used to record bead motion. Image processing of frame sequences using IPLab (version 3.2.4, Scanalytics, VA) was carried out to track the centroid position of each bead in time. The position of the bead centroid in the frame immediately preceding the onset of each force pulse was defined as the origin, and the maximal bead displacement determined at the end of the force pulse (just prior to cessation of stress) was measured relative to this origin position. In this manner, the contribution of any shift in the baseline during the course of repeated pulses was minimized. All measurements were carried out within 60 minutes of bead binding. Multiple cells sampled from the same dish also were separated from each other by at least 2 millimeters to ensure against residual effects from previous force pulses. Statistical analysis was done with SPSS software package for Windows (version 11.0.1, Chicago, IL) and with Microsoft Excel (Microsoft, Seattle, WA).

Intracellular calcium measurements

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured using calcium ratio-imaging in cells that were loaded with the calcium-binding dye, Fura-2AM (10 μM, Molecular Probes, CA) for a total of 30 min at 37°C as previously described (Grynkiewicz et al., 1985; Alenghat et al., 2004). The cells were incubated with RGD-beads during the final 10 minutes of exposure to Fura-2. The cells were then washed with PBS and analyzed on a Nikon Diaphot microscope equipped with a CCD camera. Paired Fura-2 images of cells with bound RGD-beads were captured at excitation wavelengths of 340 and 380 nm every 3 seconds. Acquisition of the paired fluorescent images was briefly interrupted after 12 seconds in order to apply mechanical forces to the cells using either the permanent magnetic needle or, for higher stress levels, a previously described electromagnetic needle (Matthews et al., 2004a).

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