Cross-linking of actin filaments by myosin II is a major contributor to cortical integrity and cell motility in restrictive environments

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Summary

Cells are frequently required to move in a local environment that physically restricts locomotion, such as during extravasation or metastatic invasion. In order to model these events, we have developed an assay in which vegetative *Dictyostelium* amoebae undergo chemotaxis under a layer of agarose toward a source of folic acid [Laevsky, G. and Knecht, D. A. (2001). *Biotechniques* 31, 1140-1149]. As the concentration of agarose is increased from 0.5% to 3% the cells are increasingly inhibited in their ability to move under the agarose. The contribution of myosin II and actin cross-linking proteins to the movement of cells in this restrictive environment has now been examined. Cells lacking myosin II heavy chain (*mhcA*$^{-}$) are unable to migrate under agarose overlays of greater than 0.5%, and even at this concentration they move only a short distance from the trough. While attempting to move, the cells become stretched and fragmented due to their inability to retract their uropods. At higher agarose concentrations, the *mhcA*$^{-}$ cells protrude pseudopods under the agarose, but are unable to pull the cell body underneath. Consistent with a role for myosin II in general cortical stability, GFP-myosin dynamically localizes to the lateral and posterior cortex of cells moving under agarose. Cells lacking the essential light chain of myosin II (*mlcE*$^{-}$), have no measurable myosin II motor activity, yet were able to move normally under all agarose concentrations. Mutants lacking either ABP-120 or α-actinin were also able to move under agarose at rates similar to wild-type cells. We hypothesize that myosin stabilizes the actin cortex through its cross-linking activity rather than its motor function and this activity is necessary and sufficient for the maintenance of cortical integrity of cells undergoing movement in a restrictive environment. The actin cross-linkers α-actinin and ABP-120 do not appear to play as major a role as myosin II in providing this cortical integrity.

Movies available online

Key words: Chemotaxis, Myosin, Force, Deformation, Under-agarose assay, Folate, *Dictyostelium*, Actin cross-linking

Introduction

Dictyostelium discoideum is a social, eukaryotic amoebae that normally moves through the soil using chemotaxis to folic acid to search for it's bacterial food source (Konijn et al., 1967). When deprived of a food source, cells in a local territory are attracted together to form a multicellular aggregate by chemotaxis to cAMP (Barkley, 1969; Bonner et al., 1969; Konijn et al., 1967). The mechanisms of chemotactic motility have been extensively investigated, and it is clear that the actin cytoskeleton provides the structural framework against which force is applied, allowing cells to change their shape. In addition, new actin filament polymerization provides at least part of the force that drives protrusions during cell motility. How this activity is polarized to allow chemotaxis is not understood; however, it is clear that cell surface receptors for chemotactic factors lead to signals in the 'front' of the cell that are translated into localized activation of the cytoskeleton (Parent et al., 1998).

The organization of these actin filaments into functional arrays and the dynamics of these arrays is also not well understood. To date, more than twenty actin-binding proteins have been discovered in *D. discoideum*. Among these are a number of actin filament cross-linking proteins, including ABP120 (Condeelis et al., 1981), α-actinin (Fechheimer et al., 1982), fimbrin (Prassler et al., 1997), cortexillins I and II (Faix et al., 1996), and a 34 kDa protein (Fechheimer and Taylor, 1984) that presumably provide rigidity to the cortex. Why the cell needs so many different actin cross-linking proteins, and what specific roles each plays in processes that involve rearrangements of the actin cytoskeleton such as chemotaxis, cytokinesis, endocytosis and phagocytosis, is unclear.

The myosin motors also play a major role in cytoskeletal function. Non-muscle myosin II assembles into minifilaments, and these filaments are able to apply force to move actin filaments relative to each other (Clarke and Spudich, 1974; Hynes et al., 1987; Sheetz et al., 1986). Myosin II minifilament assembly is regulated by the phosphorylation state of the heavy chain tail (Egelhoff et al., 1993) and the motor activity is stimulated by phosphorylation of the regulatory light chains (Griffith et al., 1987). The essential light chain appears necessary for myosin motor function since myosin lacking this protein assembles minifilaments and binds actin, but has no

measurable actin activated ATPase activity (Chen et al., 1995; Xu et al., 1996). While not generally thought of as an actin cross-linking protein, myosin II minifilaments presumably also have this capability (Wachsstock et al., 1994; Humphrey et al., 2002). Mutants lacking myosin II (*mhcA*$^-$) are able to accomplish both random and chemotactic motility; however, they move slowly and have defects in pseudopod extension (Peters et al., 1988; Wessels et al., 1988). Although *mhcA*$^-$ cells are able to aggregate, they are unable to complete the developmental program (Knecht and Loomis, 1988).

The developmental defect of *mhcA*$^-$ cells appears to be due to their inability to move in a restrictive environment (Shelden and Knecht, 1995). During early development, cells acquire surface adhesion proteins and so movement occurs while cells are continually making and breaking adhesive contacts with their neighbors as well as the substratum. Unlike movement on a planar substratum, this form of motility is analogous to the movement of metastatic cancer cells away from a primary tumor, or the extravasation of immune cells through capillaries and into a wound site. It requires cells to overcome a barrier of resistance to their movement. Movement in restrictive conditions is also important for *Dictyostelium* development. Ponte et al. showed that while development of actin-binding protein mutants on agar plates is normal, development on soil plates is defective (Ponte et al., 2000). Soil is presumably a more restrictive environment for cell motility than a planar agar surface. Myosin II seems to be essential for this multi-dimensional process of migration, apparently by providing cortical integrity, since the *mhcA*$^-$ cells became stretched and distorted when attempting to move in aggregation streams (Shelden and Knecht, 1995).

Surprisingly, cells lacking the essential light chain (*mlcE*$^-$) behave normally in this environment indicating that the motor activity of myosin is not required for motility in restrictive conditions (Xu et al., 2001). Since the environment of aggregation streams is so complex, we sought to develop a simpler and more versatile means by which cell motility in a restrictive environment could be investigated. An under-agarose folate chemotaxis assay has been developed in which cells are induced to move between a planar substratum (glass or plastic) and a layer of deformable agarose of varying stiffness (Laevsky and Knecht, 2001). Using this system, we have investigated the movement of cells lacking specific cytoskeletal proteins. Consistent with our previous results, it appears that the actin binding activity of myosin II, and not the motor activity, is required for movement and cortical stability in this restrictive environment. None of the other actin cross-linkers tested have as major a role in this process as myosin II.

Materials and Methods

Cell culture and conditions

All cell cultures were grown in 100 mm plastic Petri dishes containing 10 ml of HL-5 medium [5 g Bacto® protease peptone #2 (Difco, Detroit, MI, USA), 5 g BBL thiotone E, 10 g glucose, 5 g yeast extract, 0.35 g Na_2HPO_4, 0.35 g KH_2PO_4, 0.1 mg/ml ampicillin, 0.1 mg/ml dihydrostreptomycin, to 1 l, pH 6.7]. NC4A2 is an axenic cell line derived from the wild-type NC4 without mutagenesis (Knecht and Shelden, 1995; Morrison and Harwood, 1992). HK321 is a myosin II heavy chain null mutant (*mhcA*$^-$) derived from NC4A2 (Shelden and Knecht, 1995). *mlcE*$^-$ is an essential light chain mutant in which the light chain is replaced by the thy1 selectable marker (Chen et al., 1995; Pollenz et al., 1992). ELC+ is a cell line in which the essential light chain gene is integrated back into the genome of *mlcE*$^-$ cells in order to rescue *mlcE*$^-$ function (Chen et al., 1995; Pollenz et al., 1992). This cell line was used as a control for the *mlcE*$^-$ cells since the thy– parental of both cell lines (JH10) moves poorly in the conditions of the under-agarose assay. α-actinin and ABP-120 mutants were generated via homologous targeting in an AX2 parental line (Rivero et al., 1999). Cell lines containing the actin binding domain (ABD) of actin binding protein 120 (ABP120) fused to green fluorescent protein (ABD-GFP) (Pang et al., 1998) were used to localize F-actin. A GFP-myosin expression plasmid (Moores et al., 1996) was used to determine myosin localization.

Under-agarose assay

The under-agarose assay was performed as described previously with minor modifications (Laevsky and Knecht, 2001). 14 ml of SeaKem® GTG agarose (BMA, Rockland, ME, USA), made with SM medium (Sussman and Sussman, 1967), was poured into 100 mm plastic Petri dishes. The agarose was allowed to solidify for 1 hour at 22°C. Three 2 mm wide troughs were cut 5 mm apart with a standard razor blade (4 cm length) using a template (Fig. 1). 100 μl of 0.1 mM folic acid (Research Organics, Cleveland, OH, USA) was added to the center trough and allowed to form a gradient for 1 hour at room temperature. Cells were harvested, adjusted to 1×10^6 cells/ml for individual analysis and 1×10^7 cells/ml for population analysis. 100 μl of cell suspension was then added to the peripheral troughs.

Analysis of cell movement

Images were taken of the cell populations using a Zeiss® IM inverted microscope (Carl Zeiss, Oberkochen, Germany), Paultek Imaging Inc. CCD camera (Advanced Imaging Concepts, Princeton, NJ, USA), Scion Inc. LG3 frame grabber (MVI, Avon, MA, USA) and NIH Image software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Overall distance traveled by cells under-agarose was determined by measuring the average distance the ten front most cells in a field of view were from the trough edge, approximately 3 hours after the cells were applied to the trough. The analysis of the movement of *mhcA*$^-$ cells was done near the trough edge as soon as they could be seen to have moved underneath the agarose in order to examine cells prior to stretching and fragmentation. Individual cell speed and direction change was determined using DIAS® software (Solltech, Oakdale, IA, USA). Speed was calculated using the displacement of the centroid from frame to frame during 1-minute intervals. Direction change was measured as the absolute value of the difference in the direction of movement of the centroid from frame to frame, measured in degrees. Cross sectional area measurements were made using NIH Image software. The cross sectional area is measured as the area of the image of a cell seen using phase contrast microscopy.

Fluorescence imaging

For fluorescence imaging experiments, 0.75 ml of agarose was added to a Rose chamber (Rose et al., 1958), or 4 ml to a 60 mm glass bottom Petri dish (Willco Wells, Amsterdam, Netherlands) so that cells could be imaged through a 0.17 mm thick glass coverslip. Two troughs were cut in the Rose chamber with a 10 mm long razor blade, and the amount of cells and folate was decreased proportionally. Confocal imaging of GFP-labeled cells was performed using a Leica TCS SP2 confocal microscope system (Leica Microsystems, Heidelberg, Germany) and an MRC 600 (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a 25 mW krypton-argon laser and COMOS software.

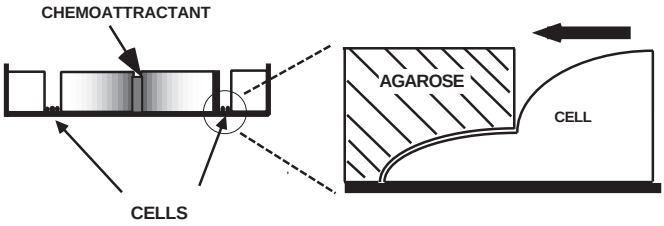

Fig. 1. Under-agarose chemotactic assay. Three parallel troughs are cut into the agarose. The center trough is filled with folate and cells are placed in the peripheral troughs. Cells then migrate towards the folate, deforming the agarose upwards while simultaneously flattening themselves. Arrow indicates direction of cell movement.

Results

Motility of cytoskeletal mutants under agarose of varying concentrations

A cell is generally able to generate a particular shape and to change shape as desired using internally generated forces. In order to do this, the cell must be able to overcome the environmental forces that resist these processes, such as membrane tension, hydrostatic pressure, fluid shear etc. The shape changes are generally accepted to be driven by the actin cytoskeleton, which together with accessory proteins make up the cell cortex. Therefore, we use the term 'cortical integrity' to refer to the structural properties of the cell cortex, i.e. its ability to deal with external or internal mechanical forces. When cells are attached to a planar substratum and moving in fluid, there is little in the way of external forces to resist cell shape changes. However, in the under-agarose chemotaxis assay, cells move between the plastic surface and a sheet of agarose (Fig. 1). As the cells move out of the trough to move up the folate gradient, they must deform the agarose upward and at the same time become flattened (Laevsky and Knecht, 2001). As the stiffness of the agarose increases, cells have more and more difficulty deforming it, until at 3% agarose, the wild-type cells can no longer move at all. This is likely to occur because at this concentration, the cells no longer have sufficient cortical integrity to deform the agarose sheet upwards. If so, then cells with reduced cortical stiffness should show defects in moving under lower concentrations of agarose compared with wild-type cells. In order to examine this possibility, several mutants that might be expected to have reduced cortical integrity were examined. ABP-120 and α-actinin are the two major actin cross-linking proteins found in the cortex of *Dictyostelium* cells (Condeelis et al., 1984). Gene disruption mutants have been isolated that lack either of these proteins and these cells have measurable but not dramatic alterations in cytoskeletal function and motility (Cox et al., 1992; Cox et al., 1996; Noegel et al., 1989). However, mutants lacking either protein showed normal movement in the under-agarose chemotaxis assay (Table 1). This result indicates that neither protein is required for under-agarose motility.

Cells lacking myosin II (*mhcA*$^-$) are able to move on a liquid covered planar surface at rates about one third of wild-type cells (Wessels et al., 1988). However, these mutants are unable to penetrate aggregation streams, which are presumed to be a viscous restrictive environment (Clow and McNally, 1999; Shelden and Knecht, 1995). In order to examine more directly whether this defect is the result of their inability to move in a restrictive environment, the under-agarose chemotaxis assay was used to examine the motility of the *mhcA*$^-$ cells. In 0.5% agarose, wild-type cells moved relatively freely out of the troughs within 1 hour and continued to do so over the next 9 hours reaching a distance of about 3000 μm from the trough (Laevsky and Knecht, 2001) (Fig. 2). In contrast, few *mhcA*$^-$ moved out of the trough, and those that did never migrated more than 500 μm from the trough (Fig. 2). Because of this, the movement of individual *mhcA*$^-$ cells was measured near the trough edge soon after exit. The speed of these cells was about two thirds of wild-type cell speed and their movement was directed toward the folate trough (Table 1). At concentrations of agarose 1% or above, the *mhcA*$^-$ cells did not move out of the troughs at all (Figs 2 and 3). In order to confirm that the inhibition of *mhcA*$^-$ movement was due to the stiffness of the agarose and not the adherence of the agarose to the plastic dish, the same experiment was performed except that the agarose layer was either rotated 180° or lifted out and placed in a fresh dish prior to cutting the troughs. The same results were obtained when the agarose was freed from the surface in this way (data not shown) indicating that it is the local deformation of the agarose and not the adhesion of the agarose to the surface that inhibits the movement of *mhcA*$^-$ cells.

Table 1. Computerized motion analysis of the movement of individual cells in the under agarose assay

	0.5% Agarose			2.5% Agarose		
	Speed (μm/min)	Direction changes	Surface area (μm^2)	Speed (μm/min)	Direction changes	Surface area (μm^2)
NC4A2	6.4±1.3	17.5±6.6	189±30.3	3.4±1.2	13.8±13	384±78.5
AX2	7.4±1.9	16.7±5.7	269±59.9	3.5±0.7	14.4±11	476±114.0
MhcA$^-$	4.2±1.2*	15.7±6.7	311±49.0*	na	na	na
ELC+	7.2±1.2	19.4±11.4	198±26.7	4.7±1.0	13.2±10.7	364±74.8
ELC–	7.2±1.1	19.4±7.6	188±17.9	3.7±0.7	11.1±12.1	317±53.0
ABP120–	5.9±0.8	17.1±6.6	270±34.7	4.0±0.6	14.6±8.4	493±90.3
α-actinin–	7.0±1.2	19.6±8.1	270±33.6	4.9±0.8	19.5±5.9	516±132.7

Individual cell speed, the number of direction changes and surface area were determined as described in the Materials and Methods section. Transmitted light images were acquired under indicated agarose concentration and quantified using DIAS® imaging software. Surface area measurements were determined using NIH software.

*Significant deviation from parental control values. The data are means±s.d. ($n \geq 10$). Differences between means were checked for significance ($P < 0.05$) with a two-way analysis of variance and the Student's *t*-test.

AX2 is the parent of 120– and 95– cell lines. NC4A2 is the parent of the *mhcA*$^-$ cells. The ELC+ serves as a control for the ELC– cell line (see Materials and Methods).

na, not applicable.

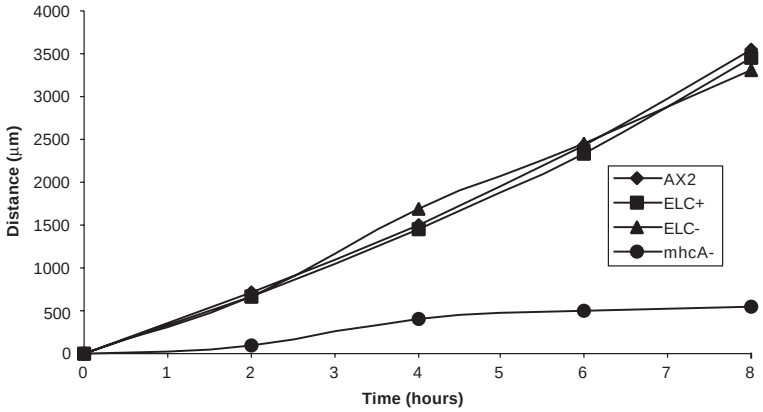

Fig. 2. Distance traveled by cells under 0.5% agarose. At each time point (indicated in hours) the distance that the ten front-most cells in one field of view had traveled from the trough edge was determined. AX2 (wild type), ELC– (essential light chain mutant), ELC+ (essential light chain mutant rescued by expression of essential light chain cDNA), and *mhcA*⁻ (myosin heavy chain null mutants).

Effects of agarose overlay on *mhcA*⁻ morphology

When moving under 0.5% agarose, the *mhcA*⁻ cells did not move as far or as fast as the wild-type cells, however, the most unusual aspect of their behavior was the dramatic elongation of the cells as they attempted to move (Figs 3, 4, 5). This behavior is reminiscent of *mhcA*⁻ cells moving in wild-type aggregation streams using the chimeric aggregation assay (Shelden and Knecht, 1995; Xu et al., 1996) (see Discussion). However, it was difficult in that assay to pinpoint the precise cause of the stretching. In the under-agarose assay, the stretched appearance of cells was found to be due to a failure of retraction of the rear of the cell body. *Dictyostelium* cells do not normally have a well-defined uropod, but this process generated a structure resembling the uropod of mammalian cells. Time-lapse analysis of cell movement indicated that the rear of the cell would often become stuck to the surface while the cell body continued to move. This uropod would eventually only be connected by a thin bridge of cytoplasm and this bridge sometimes broke as the cell body moved away (Fig. 4). Even cells that did not fragment ceased moving about 500 μm from the trough edge (Fig. 2). The posterior of wild-type cells is enriched in F-actin as shown by the bright fluorescence of the GFP-ABD120 probe in this region (Laevsky and Knecht, 2001). The posterior of *mhcA*⁻ cells is also enriched in F-actin and the probe is concentrated in the cytoplasts released from *mhcA*⁻ cells (Fig. 5). This loss of cellular actin may account for the eventual cessation of movement by these cells (Fig. 5). However, it is interesting to note that cells that do not fragment also eventually stop moving about 500 μm from the trough edge.

Previously, we showed that increasing the concentration of agarose results in an increased surface area of wild-type cells, indicating that the cell is less able to deform the agarose upward and becomes more compressed and flattened as a result (Laevsky and Knecht, 2001). If the cortex of the *mhcA*⁻ cells were flaccid, one would expect that they would have a greater surface area than wild-type cells at the same agarose concentration. The elongated appearance of the *mhcA*⁻ cells makes this comparison more complicated, however, the *mhcA*⁻ cells that were able to move under 0.5% agarose did have a significantly greater surface area than wild-type cells (Table 1).

There are two possible reasons why the *mhcA*⁻ cells might not be able to exit the troughs at high agarose concentrations. The first possibility relates to their behavior when moving under 0.5% agarose. The stretching and fragmentation indicates that the cells have trouble releasing and retracting their uropods. While this is seen to some extent when *mhcA*⁻ cells are moving in liquid media without an agarose overlay (D.A.K., unpublished observations), it is far more dramatic under agarose. Thus it is possible that at 1% and higher agarose concentrations, this problem is magnified. In this scenario, the cells would not move beyond the trough edge because once they move the cell body underneath the agarose at the edge, they become trapped because they cannot retract their uropods and move any farther. If this were the case, we would see stretched cells at the very edge of the trough. The other possibility is that the cells are unable to deform the agarose upward and bring the midbody (the thickest part of the cell) underneath, indicating a weakness in the ability of the general cell cortex to deform the

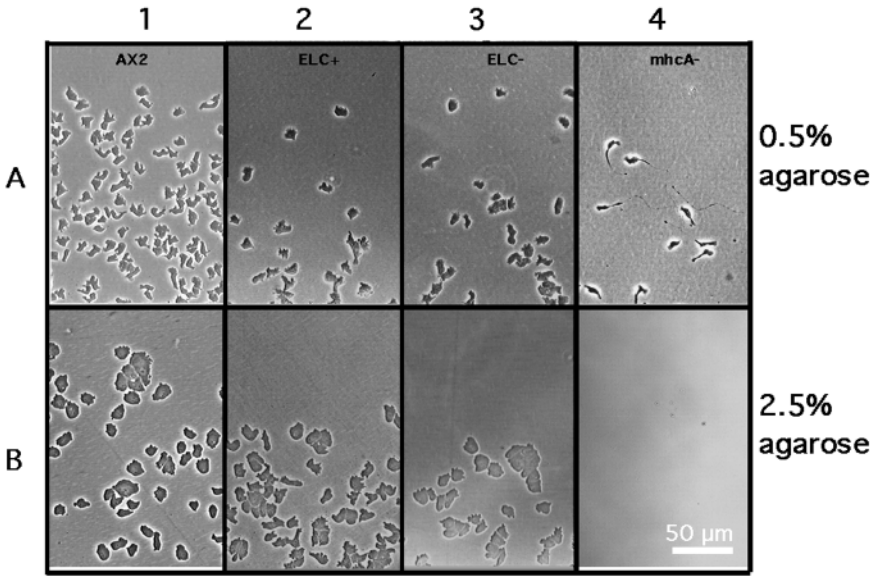

Fig. 3. Cell morphology under 2.5 and 0.5% agarose. Images of cells under agarose were acquired 5 hours after wild-type cells were added to the trough and 2 hours after *mhcA*⁻ cells were added. The trough edge interferes with the imaging, so the optical field was place about 300 μm from the edge of the trough. AX2 (wild type), ELC– (essential light chain mutant), ELC+ (essential light chain mutant rescued by expression of essential light chain cDNA), and HK321 (myosin heavy chain null mutants).

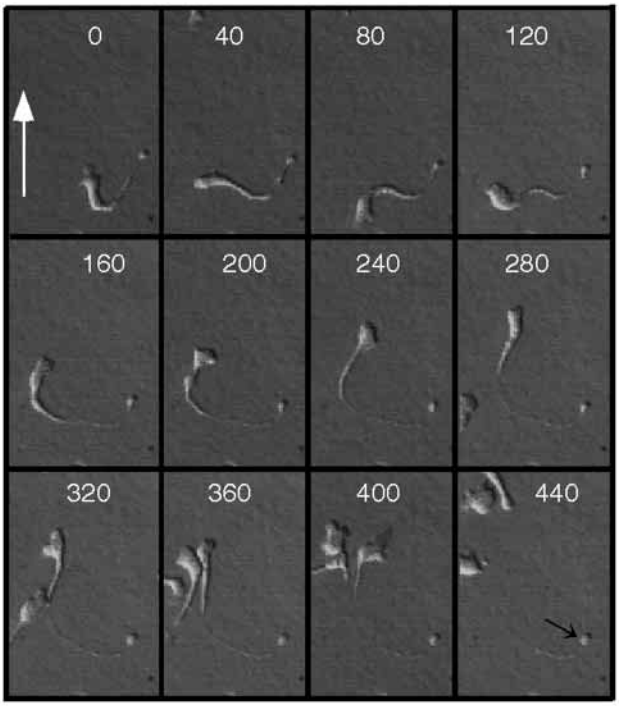

Fig. 4. Fragmentation of *mhcA*$^{-}$ cells moving under agarose. Myosin mutant cells were imaged under 0.5% agarose, within 500 μm of trough edge. The origin of the gradient and direction of cell movement is indicated by the white arrow. The montage shows images of a single cell over time as it becomes stretched and fragmented. The black arrow indicates a cell fragment left behind. Numbers indicate time in seconds. A QuickTime movie showing the fragmentation events can be viewed at http://jcs.biologists.org/supplemental/.

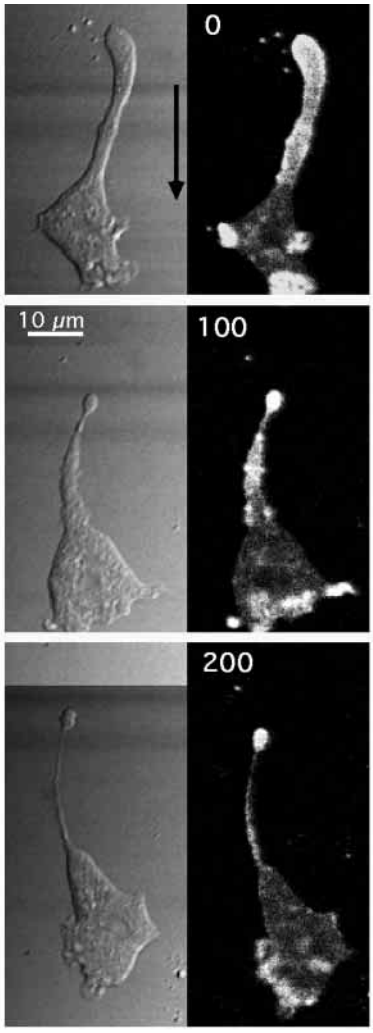

Fig. 5. GFP-ABD120 localization in *mhcA*$^{-}$ cells under agarose. Confocal images taken 100 seconds apart of GFP-ABD120 localization in *mhcA*$^{-}$ cell. The cell is moving in the direction indicated by the arrow. GFP-ABD localizes to F-actin primarily in the rear of the cell. Magnification is 63× with a z-section thickness of 0.5 μm. A QuickTime movie showing the dynamic localization can be viewed at http://jcs.biologists.org/supplemental/.

agarose. In order to distinguish between these possibilities, *mhcA*$^{-}$ cells were examined at the edge of the trough as they tried to move out under the agarose (Fig. 6). The cells moved to the edge of the trough, and then frequently extended pseudopods under the agarose sheet, but the cell body was never able to move underneath. However, the cells were able to withdraw the pseudopod and continue moving along the agarose interface. Stretched cells under the agarose at the edge were not observed indicating that uropod retraction was not the cause of the defect. This data indicates that the defect in *mhcA*$^{-}$ cells is in creating the force necessary to push the stiffer agarose out of the way and move the cell body underneath.

Essential light chain mutants move normally under agarose

Myosin II from cells lacking the essential light chain of myosin (*mlcE*$^{-}$) has actin-binding activity, but lacks ATPase motor function (Chen et al., 1995; Xu et al., 2001). In the chimeric aggregation assay, *mlcE*$^{-}$ cells moved normally and did not become elongated like *mhcA*$^{-}$ cells (Xu et al., 2001). *MlcE*$^{-}$ cells were tested in the under-agarose chemotaxis assay to see if the motor function of myosin was necessary for movement in this restrictive environment. Under all agarose concentrations tested, *mlcE*$^{-}$ cells moved the same distance and at the same speed as the control rescued cells in which the essential light chain was reintroduced into the cells (Table 1). DIAS analysis of individual cell behavior indicated that the rate of direction change was consistent with cells undergoing positive chemotaxis, as opposed to cells moving randomly (Table 1) (Laevsky and Knecht, 2001). The *mlcE*$^{-}$ mutants maintained a surface area of about 198 μm^2, similar to that of the control cells (Fig. 2, Table 1) and became comparably flatter under 2.5% agarose. Morphologically, no obvious difference was seen between the two cell lines when viewed under agarose (Fig. 3).

Localization of F-actin and myosin II during under-agarose chemotaxis

In order to determine if the localization of F-actin in *mlcE*$^{-}$ cells was altered, the GFP-ABD120 probe was introduced into the cells (Pang et al., 1998). This probe dynamically associates

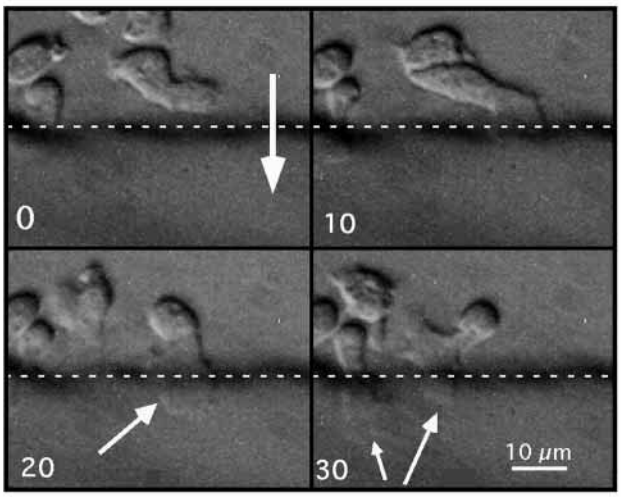

Fig. 6. *mhcA*⁻ cells are unable to move the cell body under the edge of a 2% agarose trough. *MhcA*⁻ cells were imaged at the edge of the trough. The dotted line indicates trough edge and the numbers indicate the time in seconds. The vertical white arrow points towards the origin of the chemoattractant gradient and the direction of cell movement. The cells can be seen extending pseudopods under the agarose (arrows), but the cell body remains in the trough so the cells cannot move up the gradient. A QuickTime movie showing the transition event can be viewed at http://jcs.biologists.org/supplemental/.

with F-actin filaments in live cells allowing visualization of the actin cortex. In both wild-type and *mlcE*⁻ cells moving under agarose, the probe localized to an arc around the posterior and rear edge of the cell and transiently to new protrusions at the leading edge (Fig. 7A,B). No significant difference in the localization of this probe was observed in *mlcE*⁻ cells.

Previous work has shown that myosin is distributed throughout the cortex in cells in buffer or media, but when placed under agarose, it rapidly relocalizes to the rear of the cell (Neujahr et al., 1997; Yumura et al., 1984). In order to examine the localization of myosin II during under-agarose chemotaxis, wild-type and *mlcE*⁻ cells, expressing myosin II-GFP were examined. Confocal optical slices about 0.5 μm thick were acquired every 5 seconds at a point just above the surface of the coverslip. In both cell types, myosin II is concentrated in an arc at the rear of cells undergoing under-agarose chemotaxis (Fig. 7C,D). In addition to its prevalent localization in the rear, myosin II is also found to transiently localize to small patches of the cortex at the front of the cell. No significant differences in myosin-GFP localization were observed between the wild-type and the *mlcE*⁻ cells during under-agarose motility.

In order to visualize the three-dimensional localization of myosin through the volume of the cell, 0.2 μm thick z-sections were acquired with the confocal microscope. The cells are about 4-5 μm thick under this condition, and actin (not shown) and myosin II are present in an arc or ring at the edge of the cell throughout much of this volume (Fig. 8). There is much less myosin near the dorsal surface of the cell. This conical wall of myosin (and actin) sometimes extends all the way around the cell (Fig. 8), but is frequently just in the rear half of the cortex as in the cells shown in Fig. 7. The only significant difference between wild-type and *mlcE*⁻ cells was that the later frequently had small round dots of fluorescence in the rear of the cell, which is probably results from a disassembly defect in myosin lacking the essential light chain. We hypothesize that the cortical rim of acto-myosin is the structural element that allows the cell to resist the downward pressure of the agarose and move in this environment.

Discussion

Movement of cells on a planar surface requires protrusion of the membrane at the leading edge, adhesion of this new protrusion to the surface, and retraction of the cell body. Depending upon where adhesion to the surface is concentrated, adhesions must either be released from the surface or the pull of the cell body must overcome the force of adhesion. It has been proposed that myosin II is the contractile motor that causes tail retraction. However, for many cell types, there is not a distinct 'retraction' event. Instead, the cell moves smoothly along with protrusion and retraction apparently occurring simultaneously. Also, it is clear that cells lacking myosin II can move on surfaces, albeit at a slower rate than wild-type cells (Wessels et al., 1988). If the cell is moving in a restrictive environment or a three dimensional matrix, the situation becomes even more complex as there is no longer a 'dorsal' or 'ventral' side of the cell and adhesion can take place anywhere on the

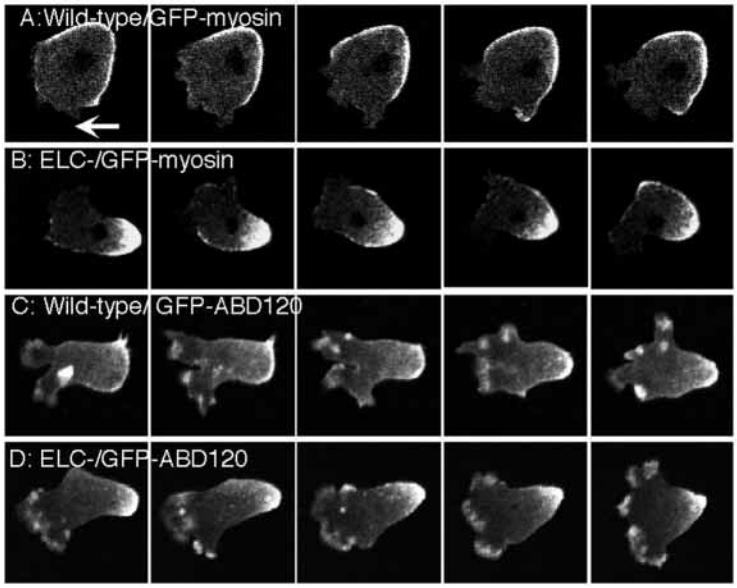

Fig. 7. Localization of F-actin and myosin II during under-agarose migration. Confocal images taken of cells moving in the direction of the arrow under 2.0% agarose. The images shown are at 30-40 second intervals in a focal plane a few microns above the substratum. (A) Wild-type cells expressing GFP-myosin II. (B) *mlcE*⁻ cells expressing GFP-Myosin II. (C) Wild-type cells expressing GFP-ABD120 to visualize F-actin dynamics. (D) *mlcE*⁻ cells expressing GFP-ABD120. The localization and dynamics of actin and myosin II are not significantly altered in *mlcE*⁻ cells. QuickTime movies of the fluorescence localization can be viewed at http://jcs.biologists.org/supplemental/.

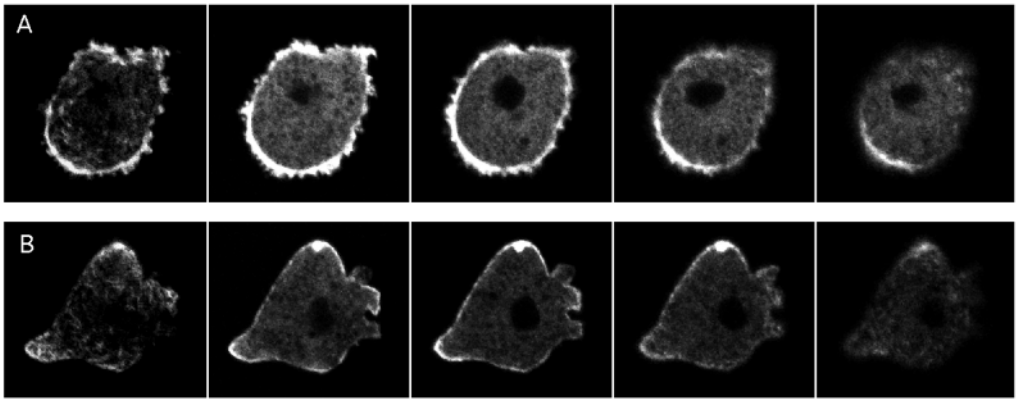

Fig. 8. Three-dimensional localization of myosin II in cells moving under agarose. *z*-series through the cells were acquired with a confocal microscope while the cells were moving under agarose. The sections shown are spaced approximately 0.2 μm apart. (A) Wild-type cells expressing myosin-GFP. (B) *mlcE*$^{-}$ cells expressing myosin-GFP. In both cases, the edge of the cell contains myosin-GFP throughout much of the 4-5 μm thickness. This would form a vertical wall of myosin and actin at the edge. The extent of this wall from front to back of the cell varies from cell to cell and over time. In many cells, the wall is primarily in an arc around the posterior of the cell as shown in Fig. 7A,B.

surface. We have begun to investigate the issue of how a cell 'squeezes' itself through a restrictive environment that provides adhesive surfaces on more than one side. In this situation, the cell is subjected to additional stresses as it must push against resisting structures or resist the pushing of other cells. We use the term cortical integrity to refer to the ability of the actin cortex to apply and resist these external forces. An example of this type of movement would be a neutrophil or macrophage extravasating through a capillary wall, or a metastatic cancer cell invading a tissue layer.

Our results indicate that myosin II is a surprisingly important player in the maintenance of cortical integrity, especially when a cell is challenged to move in a restrictive environment. Even more surprising is the finding that this action of myosin II does not appear to require the normal contractile activity. The most likely interpretation of this result is that ELC-myosin II retains the ability to bind and cross-link actin filaments and thereby the cortex, in addition to its ability to rearrange those filaments when called upon to contract. This result is consistent with rheological measurements that show that mixing myosin II with actin filaments in the presence of ADP can dramatically stiffen the matrix (Humphrey et al., 2002). How the cell might regulate this aspect of myosin function is unknown, but a precedent exists in the latch state of smooth muscle myosin where force production is not always directly linked to actin binding (Sweeney, 1998).

We, and others have previously shown that cells lacking myosin II are unable to accomplish morphogenetic movements (Clow and McNally, 1999; Shelden and Knecht, 1995). In aggregation streams the *mhcA*$^{-}$ cells were unable to move amidst the mass of adhered cells and became dramatically stretched as they tried to make and break contacts with neighboring cells in this environment (Shelden and Knecht, 1995). The defect was interpreted as a failure in cortical integrity, allowing cells to be stretched abnormally by externally applied forces. Surprisingly, cells lacking the essential light chains of myosin II behaved normally in this chimeric aggregation assay (Xu et al., 1996). In the absence of the essential light chain, myosin is found associated with the actin cortex, so presumably can bind actin, but there is minimal actin-activated ATPase motor activity (Chen et al., 1995; Xu et al., 1996). This result indicated that the motor activity of myosin II is not required for the maintenance of cortical integrity.

We envision at least three distinct force-generating steps in movement under agarose. First is the protrusive force at the leading lamella or pseudopod causing forward movement of the leading edge. Because this part of the cell is relatively thin, and there is a small space between the agarose and the planar surface, the agarose concentrations we are using are probably not especially inhibitory to this protrusion process. The second step is the upward deformation of the agarose necessary to allow the thickest part of the cell (the nuclear region) to

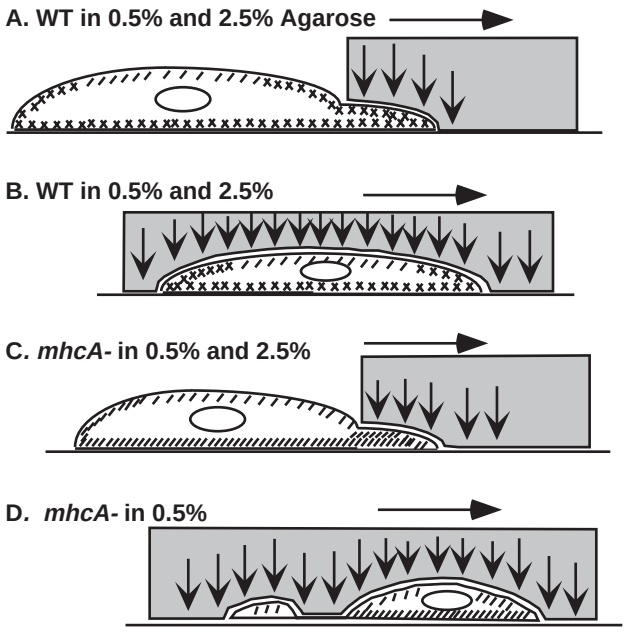

Fig. 9. Model of myosin II function in cortical stability. Cross hatching indicates cross-linking function of myosin II in a cortical acto-myosin complex. Arrows indicate direction of cell movement and downward pressure resultant from resistance to deformation of agarose. (A,B) Deformation of agarose and flattening of cell that occurs during wild-type cell migration under agarose. (C,D) Lack of cross-linking occurring as a result of loss of myosin heavy chain function. (C) The ability of the cell to extend a protrusion under the agarose similar to wild-type cells. In D, although the cell was able to retract its midbody and subsequently migrate; downward pressure imposed upon the cell by the agarose results in the uropods not being withdrawn.

squeeze underneath. This localized upward deformation as the cell crawls was shown to occur by tracking the movement of fluorescent beads embedded in the agarose (Laevsky and Knecht, 2001). As the agarose concentration is increased, it becomes more and more difficult for the cells to deform the agarose, and so movement slows down and eventually ceases around 3% agarose. The third step is the retraction of the rear of the cell. *Dictyostelium* cells do not have well defined uropods, and therefore it was not obvious that this would be separate from the translocation of the cell body. However, the finding that cells lacking myosin II have long trailing extensions of cytoplasm when moving under 0.5% agarose indicates that the detachment of the rear of the cell is indeed a separate and important issue in translocation. However, the stretching is not simply a matter of increased surface adhesion. Jay et al. examined the movement of *mhcA*$^-$ cells on surfaces of varying adhesiveness and did not observe the uropods being left behind (Jay et al., 1995). Instead the *mhcA*$^-$ cells were unable to move at all on sticky surfaces that the wild-type could still crawl on.

The inability of the *mhcA*$^-$ mutant cells to move under concentrations of agarose above 0.5% is likely to be a different problem. In this situation, the cells still make protrusions under the agarose at the trough edge, but the cell body never flattens and continues up the gradient. Thus this is not a problem of rear retraction, since the cells never get the uropod underneath the agarose. A clue to understanding this phenotype, comes from visualization of the dynamic localization of GFP-myosin in these cells. Actin and myosin are not prominent in the ventral or dorsal cortex of cells under agarose, but are enriched in the peripheral cortex, either in the rear portion as an arc, or surrounding the cell (Figs 7 and 8). This vertical ridge of acto-myosin is likely to be responsible for deforming the agarose upward and allowing the nucleus to fit underneath. In the absence of myosin II, we presume that the cortex does not have the stiffness to deform higher agarose concentrations, and so the nucleus cannot fit underneath and the cells are trapped at the trough edge.

The model in Fig. 9 explains the events proposed to occur during protrusion and retraction events. The crosshatching indicates the orthogonal network of actin filaments that lie beneath the membrane. This network would be held together by actin binding proteins, such as ABP-120, α-actinin, cortexillin, talin and myosin II. The wild-type, *mlcE*$^-$ and *mhcA*$^-$ cells are able to extend protrusions under the agarose (Fig. 9A,C). The linkage between the pseudopod and the cell body in wild-type and *mlcE*$^-$ cells is retained and the cell moves as an integral unit under the agarose (Fig. 9B). The *mhcA*$^-$ cell (Fig. 9D) is able to retract the nuclear region under 0.5% agarose, but not under 2.5% agarose. At the higher agarose concentrations, the cell apparently cannot produce sufficient force to make further progress.

The implication of these results is that the cell cortex acts to integrate the cell as a whole, and myosin II is crucial to integrating the actin cortex. The surprising result is that normal contractile activity is not needed for myosin II to carry out this function. It is possible that some contractile activity below the limit of our assays is present in ELC-myosin, and this is sufficient to allow myosin II to integrate the cortex. It has been determined that *Aspergillus nidulans* myosin I mutants with less than 1% of wild-type actin-activated MgATPase activity retain essential in vivo functions (Liu et al., 2001). However, we have shown that *mlcE*$^-$ cells cannot undergo contraction of detergent extracted cortices, which would be a direct test of contractile activity of myosin in situ (Xu et al., 2001). Another possibility is that because the actin-activated ATPase activity is lost in the *mlcE*$^-$ mutant, this mutant myosin has become a permanent actin cross-linker and this cross-linking activity replaces the normal contractile activity of myosin. This is possible, but it seems unlikely that such a dramatic change in function could allow cells to behave so normally or would allow normal organization of the actin filament network. We favor a third hypothesis, that as in smooth muscle, non-muscle myosin II is not constitutively applying force to the actin cytoskeleton, but can enter a state in which it is bound to actin like a cross-linker, while not actively engaged in the ATPase cycle. Myosin II may only be called upon to contract when the cell changes shape, as happens in cytokinesis. The *mlcE*$^-$ mutation would allow the myosin II to function in its cross-linking state, but not enter a contractile mode.

Our data indirectly indicates that the cortex of *mhcA*$^-$ is less stiff than wild-type cells. Attempts have also been made to directly measure the cortical integrity of cells using biophysical techniques. The results are contradictory and confusing. Pasternak (Pasternak et al., 1989) showed only a slight decrease (32%) in the cortical stiffness of *mhcA*$^-$ cells using a ‘cell poker’ that measured the resistance of the cell to inward deformation. Egelhoff (Egelhoff et al., 1996), using a vibrating glass rod, measured a 50% decrease in cortical stiffness in myosin II mutant cells. However, Merkel et al. (Merkel et al., 2000) used a pipette aspiration system and found a dramatic increase in the resistance of *mhcA*$^-$ cells to outward deformation from a suction pipette, indicating a stiffer cortex in the myosin II mutants. Feneberg et al. (Feneberg et al., 2001) used a microrheology technique based on colloidal magnetic tweezers to measure the viscoelastic forces within the cytoplasm. They found the apparent viscosity of myosin II null mutants was higher, also implying a stiffer cortex. Some of the discrepancies may be the result of the methodologies used. Live cell imaging of the actin cortex in cells containing the GFP-ABD120 probe shows that any time a cell makes contact with an object (another cell, a bead or an obstacle), there is a rapid accumulation of F-actin in the contact region (D.A.K., unpublished observations). Thus, application of a pipette or poker may lead to an actin polymerization response that will interfere with the measurements. By using a biological assay, we have directly evaluated the functionality of the cortex in what is to the cells, a relatively normal environment.

ABP-120 and α-actinin are, by mass, the two major actin cross-linking proteins in the cell (Condeelis et al., 1981; Condeelis and Vahey, 1982). Thus it is surprising that mutants lacking these proteins had no altered phenotype in this assay. This result indicates that not only is myosin II important for cortical integrity, but that so far, it is the single most important protein providing this function. Clearly, cells lacking myosin II have some cortical integrity or they would not be able to move at all. This residual cortical integrity is presumably supplied by the myriad of other actin cross-linkers or the rheological properties of actin filaments themselves.

Our model is not intended to suggest that cells do not require the motor activity of myosin II. Mutants lacking the essential light chain (and thus motor activity) are unable to divide in

suspension and have defects in multicellular development (Chen et al., 1995). In addition, we have previously shown that myosin II contractile activity is needed for cells to generate shape in suspension or to elongate vertically off a surface (Shelden and Knecht, 1996) and the essential light chain mutants are defective in both functions (Xu et al., 2001). Our model, therefore, proposes that myosin plays a major role in maintaining the physical integrity of the actin cortex, and that its function can be separated into contractile and actin-binding activities.

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