Actin-based propulsive forces and myosin-II-based contractile forces in migrating Dictyostelium cells

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Summary
It has been suggested that myosin II exerts traction forces at the posterior ends and retracting pseudopodia of migrating cells, but there is no direct evidence. Here, using a combination of total internal reflection fluorescence (TIRF) microscopy and force microscopy with a high spatial resolution of ~400 nm, we simultaneously recorded GFP-myosin II dynamics and traction forces under migrating Dictyostelium cells. Accumulation of filamentous myosin II and a subsequent increase in traction forces were detected in pseudopodia just before retraction. In the case of motorless myosin II, traction forces did not increase after accumulation, suggesting that the source of the retraction force is the motor activity of accumulated myosin II. Simultaneous recording of F-actin and traction forces revealed that traction forces were exerted under spot-like regions where F-actin accumulated. Cells migrated in a direction counter to the sum of the force vectors exerted at each spot, suggesting that the stress spots act as scaffolds to transmit the propulsive forces at the leading edge generated by actin polymerization.

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Key words: Amoeboid movement, Cell migration, Pseudopod

Introduction
Cell migration plays an essential role during the development of most organisms (reviewed in Lauffenburger and Horwitz, 1996; Ridley et al., 2003; Raftopoulos and Hall, 2004). In adults, cell migration continues to play an important role in processes such as wound healing (Reid et al., 2005; Zhao et al., 2006) and immune system function (Parent, 2004). It is well known that cell migration is a complex process mediated by dynamic changes in the actin-myosin cytoskeleton. It is now generally thought that actin polymerization at the leading edge provides a crucial driving force for extension of growth cones (Dent and Gertler, 2003; Kalil and Dent, 2005), keratocytes (Svitkina et al., 1997; Pollard and Borisy, 2003; Jurado et al., 2005), fibroblasts (Wang, 1985; Gaibraith and Sheetz, 1997), neutrophils (Torres and Coates, 1999; Parent, 2004) and Dictyostelium cells (Yumura et al., 1984; Yumura, 1996a; Yumura and Fukui, 1998; Parent, 2004), whereas the detachment and retraction of the rear of the cell from the substratum is thought to be induced by contraction through myosin-II-dependent processes in Dictyostelium cells (Yumura et al., 1984; Yumura and Kitanishi-Yumura, 1990; Yumura, 1993; Small, 1989; Jay and Elson, 1992; Jay et al., 1995; Uchida et al., 2003) and fibroblasts (Chen, 1981; Gaibraith and Sheetz, 1997).

If this is the case, then substratum contact sites in the anterior portions of migrating cells should function as anchorages or scaffolds to allow pushing of the leading edge. This pushing should occur by counter forces generated by actin polymerization and exerted onto the substratum in a direction opposite to that of extension. At the posterior ends of migrating cells and the tips of retracting pseudopodia, by contrast, traction forces in the same direction as the retraction should be exerted onto the substratum by myosin II contraction before detachment from the substratum. However, the relationship between myosin II dynamics and traction forces has never been demonstrated directly because of the difficulty in simultaneously recording traction forces and myosin II dynamics in migrating cells. In this study, we aimed to examine this relationship directly.

To determine the mechanical interactions between migrating cells and the substratum, Dembo and colleagues (Dembo et al., 1996; Dembo and Wang, 1999) proposed procedures to convert the measurement of elastic substratum deformation under migrating cells into traction stresses using finite element methods; this technique was designated ‘force microscopy’ and was used to map forces in migrating fibroblasts (Beningo et al., 2001; Munevar et al., 2001a; Munevar et al., 2001b; Munevar et al., 2004; Shiu et al., 2004; Curtze et al., 2004). Force microscopy revealed dynamic traction stresses at the leading edges of migrating fibroblasts (Munevar et al., 2001a; Munevar et al., 2001b).

In general, the magnitude and spatial distribution of traction forces are related to cell shape and the mode of movement (Oliver et al., 1999). Keratocytes are known as fast-moving cells with a consistent fan shape, whereas fibroblasts are slow-moving irregularly shaped cells. Traction forces were not detected at keratocyte leading edges but, rather, at the lateral cell edges, where they were oriented perpendicular to the direction of cell motion and towards the center of the cell (Lee et al., 1994; Doyle et al., 2004; Doyle and Lee, 2005; Jurado et al., 2005).

Dictyostelium cells are known as fast-moving irregularly shaped cells (Wessels et al., 1994; Heid et al., 2005). Uchida et al. (Uchida et al., 2003) and Ladam et al. (Ladam et al., 2005) found that Dictyostelium cells exert forward traction forces at their front and backward forces at their rear. Tsujikawa and colleagues (Tsujikawa et al., 2004) also detected forward traction stresses in migrating Dictyostelium cells. Recently, Lombardi et al. (Lombardi et al., 2007) mapped traction stresses in migrating vegetative Dictyostelium cells. They found a lack of traction stress at the front but high stress at the rear of the cells, in contrast to Uchida et al. (Uchida et al., 2003),
To answer these questions and investigate the relationship between molecular dynamics and traction forces, we simultaneously recorded the distributions of F-actin and traction stresses using confocal microscopy with a stress-recording spatial resolution of ~400 nm. Rearward traction stresses were detected under spot-like regions in the front of cells where F-actin accumulated. Cells migrated in a direction counter to the sum of the force vectors at each spot, suggesting that counter forces to F-actin-based propulsion are exerted at the spots. Moreover, we recently developed a new method for simultaneously recording molecular dynamics and traction stresses under migrating cells using TIRF microscopy (Iwadate and Yumura, 2008). Using this new method, we recorded the accumulation of filamentous myosin II and subsequent increase of traction stresses under pseudopodia just before their contraction, directly demonstrating that the source of the retraction force is the accumulated myosin II.

**Results**

**Rearward traction stresses at ‘stress spots’ and forward stresses at the posterior edge**

Aggregation-competent wild-type cells expressing GFP-ABD120k were dispersed on a gelatin substratum with fluorescent beads attached as illustrated in Fig. 1A,B. From the displacement of the beads by migrating cells, we calculated the stresses in the substratum using the Pascal-scale and visualized them in pseudocolor. It should be noted that the calculated stresses are not representative of all forces generated by the cell but the forces transmitted to the substratum. In a stress map of a typical wild-type cell just before posterior edge contraction, several traction stress peaks with rearward directions could be observed as ‘stress spots’ in the anterior and middle regions (white arrows in Fig. 1D). By contrast, a large stress with a forward direction was observed at the posterior edge (white arrowhead in Fig. 1D). Interestingly, the magnitude of the forward stress under the posterior edge often exceeded that of the rearward stress. If the posterior edge stress is exerted passively, as indicated by Uchida et al. (Uchida et al., 2003), Tsujioka et al. (Tsujioka et al., 2004), Ladam et al. (Ladam et al., 2005) and del Alamo et al. (del Alamo et al., 2007), or not, as suggested by Lombardi et al. (Lombardi et al., 2007)? Second, if not, then what is the source of myosin-II-independent traction stress at the rear of myosin-II-null cells?

**Stress spots act as scaffolds to transmit the counter force of actin-mediated propulsion at the leading edge**

In the measurements using elastic substrata, it is plausible that stress spots are generated at cell-substratum attachment sites. In

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**Fig. 1.** Typical stress map under a migrating *Dictyostelium* cell. (A) Wild-type *Dictyostelium* cells (strain AX2) or myosin-II-null cells (strain HS1) transformed with GFP-myosin-II, E476K mutant myosin II or ABD120k constructs were placed on elastic silicone or gelatin substrata embedded with orange- or red-fluorescent marker beads (200-nm or 20-nm diameter). The fluorescence of GFP and the marker beads was imaged simultaneously under TIRF or confocal microscopy. Migrating cells cause strains in the elastic substratum and displacements of the beads in the substratum (arrows). (B) A typical image of 200 nm marker beads under confocal microscopy. The outline of a migrating cell is superimposed as a white line. Displacements of ~300 marker beads under and surrounding a migrating cell were measured. (C) The coordinates of each bead and their displacements were transformed to those of each node of a triangle mesh. The length of the sides of each triangle is ~400 nm (~8 pixels). The stresses in the surface of the substratum were calculated using original software based on the triangle finite element method (see Materials and Methods). (D) Stress map for a migrating wild-type cell on a gelatin substratum. The distributions of stresses in the substratum were visualized by pseudocolor. The direction of the strain in the substratum at each small white circle is indicated by a white bar. The length of the white bars is three times as long as the strain. The yellow allow indicates the direction of cell migration. Large rearward traction stresses emerged as ‘stress spots’, indicated by the two white arrows. Forward stresses emerged at the posterior edge, as indicated by the white arrowhead. Numerical values in the kPa scale are indicated near each arrow and arrowhead. Bars, 4 μm (B-D).

*Dictyostelium* cells, an actin-containing structure called the ‘actin focus’ appears on the ventral membrane (Yumura and Kitaniishi-Yumura, 1990) and might act as a focal contact (Uchida and Yumura, 2004). To elucidate the relationship between traction forces and actin, we observed the dynamics of F-actin and stresses under migrating cells using confocal microscopy. F-actin was visualized by GFP-ABD120k. Figs 2 and 3 show consecutive images of GFP-ABD120k and traction stresses under wild-type (Fig. 2A) and myosin-II-null cells (Fig. 3A). Large traction stresses were observed under the stress spots (a ‘-c’ in Fig. 2A) in the anterior and middle regions of the wild-type cells with rearward directions relative to...
the direction of migration. In many cases, F-actin accumulated at the same time or just before formation of the stress spots (a-c in Fig. 2A). The distance between the center of each F-actin accumulation spot and corresponding stress spot was very close (0.32±0.22 μm, n=16 from five cells, left column in Fig. 3C). The value of the stresses at each spot reached a maximum 3.9±3.5 seconds (n=16 from 5 cells, left column in Fig. 3D) after the fluorescence of GFP-ABD120k at the center of each F-actin accumulation reached a maximum. Interestingly, the position of each stress spot never changed until its disappearance, and the stress spots persisted after the disappearance of accumulated F-actin. These observations indicate that forces generated in migrating cells are transmitted to the substratum at spots where F-actin accumulates.

Close contact between F-actin accumulation spots and the substratum was confirmed by simultaneous fluorescence and interference reflection microscopy (IRM). The darker area of the IRM image indicates that the cell surface is closer to the substratum. As shown in Fig. 2B, the positions where F-actin accumulated (d-g in Fig. 2B) coincided well with the dark spots surrounded by white rings (d'-g' in Fig. 2B) that are a typical feature of actin foci (Uchida and Yumura, 2004).

Next, to clarify whether myosin II generates rearward stress at the stress spots, stresses were observed under myosin-II-null cells (Fig. 3). Myosin-II-null cells exerted rearward stresses under regions where F-actin accumulated (a-c and a'-c' in Fig. 3A), as in wild-type cells, and close contact of the cell surface to the substratum was again confirmed by IRM (d-f and d'-f' in Fig. 3B). The distances between the center of each F-actin accumulation spot and corresponding stress spot were very close (0.34±0.19 μm, n=15 from five cells, right column in Fig. 3C). The value of the stresses at each spot reached a maximum 4.2±3.3 seconds (n=16 from five cells, right column in Fig. 3D) after the fluorescence of GFP-ABD120k at the center of each F-actin accumulation reached a maximum. These values are almost the same as those of the wild-type cells. The maximum magnitude and duration for each stress spot with a rearward direction is shown in Fig. 3E,F. There was no significant difference in maximum stress between wild-type cells (0.64±0.27 kPa, n=29 in five cells) and myosin-II-null cells (0.71±0.19 kPa, n=24 in three cells, Fig. 3E). There was also no significant difference in spot duration time between wild-type cells (23.6±6.1 seconds, n=7 in five cells) and myosin-II-null cells (26.2±9.6 seconds, n=6 in three cells, Fig. 3F). Therefore, myosin II does not contribute to rearward traction stresses, and these stresses might reflect the counter forces of actin-mediated propulsion.

As the stress spots usually did not emerge at the leading edge, it is difficult to determine directly the relationship between stress spots and actin polymerization at the leading edge. However, the relationship can be estimated indirectly by comparison of the average stress vector at all spots and migration direction, using myosin-II-null cells to account for the influence of myosin II. If the stress spots act as counter-force-transducing scaffolds, the cells should migrate in a direction opposite to the average stress vector.

Fig. 2. Colocalization of rearward traction stress spots and F-actin accumulation sites, and close contact of the cell surface to the substratum in wild-type cells. (A) Simultaneous recording of fluorescence of GFP-ABD120k (left) and traction stresses (right) of a migrating wild-type cell transformed with GFP-ABD120k under confocal microscopy. Stress spots (a-c) appeared at locations where F-actin accumulated (a-c) in the anterior and middle regions of the cell. The typical sequential stress map shown was made from three migrating cells randomly selected from 11 cells. (B) Simultaneous recording of fluorescence of GFP-ABD120k (left) and IRM imaging (right) of a migrating wild-type cell transformed with a GFP-ABD120k construct under confocal microscopy. Dark spots surrounded by white rings (d'g') appeared at locations where F-actin accumulated (d-g). The white ring surrounding a dark spot is a feature of focal contacts in Dictyostelium (Uchida and Yumura, 2004). The time-courses are indicated for each picture of GFP-ABD120k. Yellow arrows at 0 seconds in panels A and B indicate the direction of cell migration. Bars, 4 μm.
The trajectory of a migrating myosin-II-null cell and the average of strain vectors under the cell are shown in Fig. 4A (see also supplementary material Movie 1). The black line shows the trajectory of the centroid of a migrating myosin-II-null cell over a period of 400 seconds. Each blue line emerging from the trajectory shows the strain vectors in the substratum under the cell. The images a-f in Fig. 4A show the stress maps of the cell positioned at points a-f in Fig. 4A. Sequential images of the cell outline at the moment when the cell was at the positions marked with asterisks in Fig. 4A are shown in Fig. 4B. The migration speed of this cell was 2.70 μm/minute. The average speed of myosin-II-null cells on an elastic substratum was 2.58±0.35 μm/minute (n=7). By contrast, the average speed of wild-type cells was 6.87±1.63 μm/minute (n=11). These results indicate that the myosin-II null cell migrated in a direction opposite to the average traction stress, suggesting that the stress spots act as scaffolds to transmit counter forces for actin-mediated propulsion at the leading edge.

Myosin II contributes to forward traction stresses at the posterior edge

Fig. 5A (see also supplementary material Movie 2) shows consecutive maps of traction stresses under a migrating wild-type cell. The cell migrated towards the upper left. The cell retracted its posterior, exerting a large traction stress on the substratum (white arrow in Fig. 5A). It is noteworthy that there was no equivalent rearward stress, suggesting that the forward stress is not only the counter force of propulsive rearward forces at the stress spots but also the sum of the counter force and an active force at the forward edge. Time-courses of the magnitudes of each traction stress and the length of the posterior region are summarized in Fig. 5B. The magnitudes of posterior edge traction stresses (red lines in Fig. 5B) increased strongly at the beginning of retraction, after which the posterior edges retracted quickly. Interestingly, traction stresses began to decrease before retraction was completed, suggesting that the detachment from the substratum took place during retraction.

Myosin II localizes to the posterior of migrating Dictyostelium cells (Yumura et al., 1984). To determine whether myosin II is the source of the actively generated forward traction stress, the magnitudes of forward and rearward stresses at stress spots were compared between wild-type and myosin-II-null cells. Fig. 5C shows the values of the traction stresses at each stress spot in the anterior (Fig. 5C, 0.40±0.24 kPa, n=12 in five cells) and at the posterior edges (Fig. 5C, 0.70±0.21 kPa, n=16 in five cells) of wild-type cells. All values were measured when posterior edge stress peaked. The magnitude of the forward traction stresses significantly exceeded that of the rearward stresses (P<0.001). However, in myosin-II-null cells, the magnitude of the forward traction stresses (Fig. 5D, 0.58±0.21 kPa, n=5 in three cells) never exceeded that of...
the rearward stresses (Fig. 5D, 0.79±0.29 kPa, n=8 in three cells, P>0.05). These observations strongly suggest that myosin II contributes to forward active stresses at the posterior end of wild-type cells.

Myosin II contributes to traction stresses exerted at the tips of retracting pseudopodia

Freely migrating Dictyostelium cells often elongate pseudopodia in the lateral direction. These pseudopodia seem to be retracted through a myosin-II-dependent process similar to that operating at the posterior edge. Thus, we observed the traction stresses under these lateral pseudopodia. Intense traction stresses were detected not only under the posterior edge but also under the tips of the retracting pseudopodia in wild-type cells (right images in Fig. 6A,B), where F-actin also accumulated (left images in Fig. 6A,B). Fig. 6A’ and 6B’ show the time-courses of traction stresses under the tips of each pseudopod, with the lengths shown in Fig. 6A.B. The magnitude of the traction stresses reached a maximum just after the beginning of retraction in a manner similar to that occurring at the posterior edges (Fig. 5). In myosin-II-null cells (Fig. 6C-F), increased traction stress was scarcely detected at the tips of pseudopodia (Fig. 6C’-F’), although F-actin still accumulated (left pictures in Fig. 6C-F). Only ambiguous increases in traction stresses could be observed at the tips of large pseudopodia (arrow in Fig. 6F’). These weak traction stresses might be produced by means of the tension of the cell cortex, including the cell membrane and cytoskeleton, in the absence of a myosin-II-mediated process. The time-courses of the length of pseudopodia in Fig. 6A,B and C-F are the same as those dealt with later in Fig. 7A and Fig. 8B, respectively.

The timing of pseudopod elongation and retraction was compared between wild-type and myosin-II-null cells (Fig. 6G). Elongation time was defined as the time at which the length of each pseudopod reached 80% of the maximum length. Retraction time was defined as the time from attaining the maximum length until returning back to the original length. Although there was no significant difference in elongation time between wild-type and myosin-II-null cells, the retraction time in wild-type cells was significantly shorter than in myosin-II-null cells, suggesting that myosin II mediates the pseudopod retraction.

Myosin II motor activities are required for traction force exertion in retracting pseudopodia

In the above experiments, myosin-II-mediated traction forces were studied indirectly using myosin-II-null cells. During pseudopod retraction, myosin II has been shown to accumulate (Yumura, 1996b; Moores et al., 1996). We therefore next investigated the dynamics of myosin II filaments and traction stresses by simultaneous combination of TIRF and force microscopy using myosin-II-null cells expressing GFP-labeled myosin II.

Myosin II filaments began to accumulate in pseudopodia during elongation (0–40 seconds in Fig. 7A, see supplementary material Movie 3). The accumulation of myosin II filaments was followed by increased traction stress in the substratum under the pseudopod (40–80 seconds in Fig. 7A). Pseudopodia began to retract at the same time as the start of the increase in traction stress (40 seconds in Fig. 7A). The time-courses of pseudopod area, GFP-labeled myosin II fluorescence intensity and average traction stress are summarized in Fig. 7B.C. Increased pseudopod area was followed by the accumulation of myosin II (Fig. 7B), and traction stress reached a maximum during pseudopod retraction (Fig. 7C). These observations directly demonstrate that the accumulation of myosin II in pseudopodia mediates their retraction.
the time-course is indicated in each picture. The yellow arrows indicate the direction of cell migration. Posterior cell edges are indicated by a white arrow (b at 27 seconds). The cell retracted its posterior edges quickly at 0-84 seconds after forward traction stress reached a maximum. Three sequential stress maps were made from 11 migrating cells, and a typical sequence is shown. Bar, 4 μm. (B) Time-courses of the values of traction stresses (red) and the length of the posterior region (the length of the perpendicular lines from the posterior edge to the white lines at 0 seconds in panel A). The magnitudes of traction stresses at the posterior edges (red lines) strongly increased at the beginning of posterior edge retraction. After the increase in traction stress, the posterior edges retracted quickly. (C) Average values of forward traction stresses at the posterior edges (n=12 in five cells) and rearward traction stresses at the stress spots in the anterior and middle regions (n=16 in five cells) in wild-type cells when the forward traction stress reached a maximum. The magnitude of the forward traction stress at the posterior edge significantly exceeds that of the rearward stresses at stress spots in the anterior and middle regions (P<0.001). (D) Average values of forward traction stresses at the posterior edges (n=5 in three cells) and that of rearward traction stresses at the stress spots in the anterior and middle regions (n=8 in three cells) in myosin-II-null cells when the forward traction stresses at the posterior edges reached a maximum. There was no significant difference between rearward and forward stresses (P>0.05).

To examine whether the motor activities of myosin II are required for the generation of traction forces, the dynamics of the E476K motorless myosin II filament and traction stresses in silicone substrata were simultaneously observed using myosin-II-null cells expressing GFP-E476K myosin II. E476K myosin II filaments began to accumulate in pseudopodia (0–24 seconds in Fig. 8A,B) in addition to wild-type myosin II. Traction stress slightly increased only under the tip of the pseudopod (arrows in Fig. 8A) in contrast to wild-type myosin II. These weak traction stresses might be produced by means of the tension of the cell cortex, including the cell membrane and cytoskeleton, in the absence of a myosin-II-mediated process. These results indicate that myosin II motor activities are required for the generation of traction stresses during pseudopod retraction.

Discussion
In this study, we examined the spatiotemporal patterns of cell-substratum mechanical interactions and their relationships with molecular dynamics. The maximum value of rearward traction stress measured was 0.64±0.27 kPa for wild-type Dictyostelium cells, which agree with the data of Tsujioka and colleagues (Tsujioka et al., 2004). However, the traction stresses under fibroblasts and keratocytes are slightly different. Muneev and colleagues (Muneev et al., 2001a; Muneev et al., 2001b) observed dynamic traction stresses at the leading edges of migrating fibroblasts, with maximum values of several tens of kPa. Doyle and colleagues (Doyle et al., 2004; Doyle and Lee, 2005) and Jurado and colleagues (Jurado et al., 2005) mapped traction stresses under migrating keratocytes and estimated the maximum value to be several kPa at the lateral edges. The traction stresses exerted by fibroblasts and keratocytes are ~100-fold and tenfold larger, respectively, than those exerted by Dictyostelium cells; these differences might arise owing to differences in cell shape and mode of movement.

Most rearward traction stresses emerged at spots where accumulation of F-actin took place (Fig. 2) and did not involve myosin II (Fig. 3C-F). These findings indicate that traction stresses are generated as a result of actin polymerization at the stress sites or at the leading edge of the cell. The stress spots persisted after the disappearance of accumulated F-actin (Fig. 2A), suggesting that stress spots do not directly generate the traction force even though there is tight contact between the cell and the substratum. Rather, the stress spots might apply the counter force for actin polymerization at the leading edge. However, stress spots did not always emerge near the leading edge, as freely migrating Dictyostelium cells randomly extend pseudopodia not only to the substratum but also to the upper free space. Therefore, it is difficult to correlate each stress spot to each pseudopod directly. Thus, we compared the migration direction of cells with the summation of strain vectors of stresses during migration. As expected, cells continued to migrate in a direction counter to the average force vector at each spot (Fig. 4). This result suggests that F-actin assemblies in the pseudopod are connected to the actin meshwork in the cortex of the cell body and the stress spots emerge in the meshwork and act as scaffolds to transmit the counter force for the actin-mediated propulsion force at the leading edge.

Uchida and Yumura (Uchida and Yumura, 2004) detected the deformation of elastic substrata even under very small actin foci (<1 μm), although we could not detect traction stresses in foci of the same size. To calculate accurately the stresses in elastic substrata using finite element methods, the strain of the substrata should be at the smallest detectable level. Thus, we adjusted the Young’s modulus of gelatin and silicone substrata to be 2.8 kPa and 1.0 kPa, respectively. The accumulated F-actin colocalized with the close contact points between cells and the substrata (Fig. 2B and Fig. 3B). It is therefore likely that the accumulated actin foci make contacts between the cell surface and substrata that are strong enough to exert traction forces even after F-actin has dispersed (Fig. 2A and Fig. 3A). In this study, the positions of some stress spots...
and corresponding F-actin accumulation spots did not completely match, as in areas a and a’ in Fig. 2A. This might be attributable to the slight difference in the positions between the F-actin accumulation and the corresponding contact site of the cell surface to the substratum. Furthermore, a few stress spots emerged without an immediately preceding F-actin accumulation there. The contact sites of the cell surface to the substratum of these stress spots might be produced by an F-actin-independent process. These facts raise the possibility that, in order to exert traction stresses to the substratum for cell migration, only the strong contact between the cell surface and the substratum might be required as a scaffold, and it can be produced through an F-actin-independent process, although F-actin accumulation is mainly responsible for it.

We detected forward stresses at the posterior edge of the cells; if these stresses were induced passively by the frontal towing force, they should never exceed the rearward stresses at the leading edge. However, in the case of wild-type cells, forward stresses at the posterior edge often exceeded rearward traction stresses at the leading edge (Fig. 1D; Fig. 5A-D). These large forward stresses were also detected under the tips of retracting pseudopodia, suggesting that some active forces are exerted at the posterior edges of the cells and the tips of retracting pseudopodia. Jay and colleagues (Jay et al., 1995) predicted that myosin-II-mediated force should induce detachment from the substrata. If this is the case, then strong forward stresses should be detected just before the detachment. In the present study, we directly visualized the relationships among myosin II, traction forces and cell movement by using a combination of TIRF and force microscopy (Iwadate and Yumura, 2008), which revealed that, just before pseudopod contraction, filamentous myosin II and traction stresses accumulated at the tips of pseudopodia (Fig. 7). The migration speed of myosin-II-null cells on elastic substrata (2.58±0.35 μm/minute, n=7) was significantly slower than that of wild-type cells (6.87±1.63 μm/minute, n=11). These observations strongly suggest that the myosin-II-mediated force at the posterior edge contributes to the ability to migrate.

Uchida et al. (Uchida et al., 2003), Tsujioka et al. (Tsujioka et al., 2004), del Alamo et al. (del Alamo et al., 2007) and our group detected rearward traction stresses under migrating Dictyostelium cells, although Lombardi et al. (Lombardi et al., 2007) did not. Tsujioka et al. (Tsujioka et al., 2004) noted that the force-field of traction stresses was not uniform, showing a few regions with relatively large forces that were mostly stationary with respect to the substratum. This finding matches the distribution of ‘stress spots’ in the present study. The discrepancy in detection of rearward stresses in the above-mentioned studies might be due to differences in the types of cells used for measurements. Uchida et al. (Uchida et al., 2003), Tsujioka et al. (Tsujioka et al., 2004) and our group used aggregation-competent cells, whereas Lombardi et al. (Lombardi et al., 2007) used vegetative cells. The migrating speed of vegetative cells (1.13 μm/minute) (Lombardi et al., 2007) is much lower than that of aggregation-competent cells, suggesting that myosin II plays a crucial role in the ability to migrate and contribute to rearward traction stresses.

**Fig. 6.** Myosin II contributes to traction stresses under the tips of retracting pseudopodia in wild-type cells. (A,B) Fluorescence of GFP-ABD120k (left) and stress maps (right) under two typical pseudopodia from five migrating wild-type cells. Large traction stresses were observed under the tips of retracting pseudopodia where F-actin accumulated. (A’,B’) Time-courses of traction stresses (red lines) under the tips of each pseudopod (A,B) and pseudopod lengths (blue lines). (C-F) Fluorescence of GFP-ABD120k (left) and stress maps (right) under four typical pseudopodia from three migrating myosin-II-null cells. Large traction stresses were not observed under the tips of retracting pseudopodia where F-actin accumulated. (C’-F’) The time-courses of pseudopod lengths and traction stresses just under the tip of each pseudopod. Blue and red lines indicate pseudopod lengths and stresses, respectively. Although a small rise in stress took place under the tip of a large pseudopod (arrow in F’), stresses under the pseudopod tips were unchanged typically between the elongation and retraction. (G) The average periods of elongation and retraction of pseudopodia in wild-type (n=5 in three cells) and myosin-II-null cells (n=7 in three cells). Although there was no significant difference in elongation time between wild-type and myosin-II-null cells, the retraction time of pseudopodia in wild-type cells was significantly shorter than in myosin-II-null cells. Bars, 1.5 μm. The look-up table (LUT) of A and B is indicated at the right side of B, and the LUT of C-F is at the right side of D.
passive force \( ff \), with contraction taking place at the posterior edge of the cell might also exert an active force \( 'Fm' \) in Fig. 9B) triggered by the cell with motorless myosin II (arrows in Fig. 8A). Myosin II at the posterior end, as shown at the tip of a retracting pseudopod of this behavior passively induces forward force \( 'ff' \) at the stress spots as a counter force for elongation of pseudopodia in the anterior of the cell. A part of the actin-mediated \( 'rf' \) at the stress spots b in Fig. 2A, and at the inside of the cell, as shown in the stress spot a in Fig. 2A. These results suggest that there are two kinds of rearward traction stresses as follows. (1) The stress emerging at the tip of pseudopodia. The pseudopod, which expanded away from the substratum, captures the substratum at its tip. Then, it exerts rearward traction stresses onto the substratum by means of the contracting force of the expanded cortex. (2) Counter stress generated by actin polymerization in the expanding pseudopod. This stress should be exerted at the scaffold for actin polymerization. The scaffold should be separate from the tip of an expanding pseudopod, such as at the base of it or at the inner region of the cell.

We propose here a model for the migration of fast-moving irregularly shaped cells (Fig. 9). Munevar and colleagues (Munevar et al., 2001a) proposed a frontal towing model for the migration of fibroblasts, which are slow-moving irregularly shaped cells. Our model includes a frontal towing and rear contraction mechanism; it is essentially Munevar’s model with an added myosin II contractile device \( 'p' \) in Fig. 9A) at the posterior edge. Actin polymerization (red-filled circles in Fig. 9A) produces a pushing force \( 'F' \) (Fig. 9B) at the leading edge \( 'a' \) in Fig. 9A) and a rearward force \( 'rf' \) at the stress spots as a counter force for elongation of pseudopodia in the anterior of the cell. A part of the actin-mediated propulsion force \( 'F' \) in Fig. 9A) is transmitted to the posterior edge of the cell through the elastic transition zone \( 'e' \) in Fig. 9A), and this behavior passively induces forward force \( 'ff' \) in Fig. 9A) at the posterior end, as shown at the tip of a retracting pseudopod of the cell with motorless myosin II (arrows in Fig. 8A). Myosin II might also exert an active force \( 'Fm' \) in Fig. 9B) triggered by the passive force \( ff \), with contraction taking place at the posterior edge (Fig. 9B). Merkel and colleagues (Merkel et al., 2000) showed that, in response to the aspiration of a small part of the cell surface by a micropipette, myosin II accumulated at the tip of the aspirated cell lobe. This suggests the possibility that myosin II might accumulate in response to mechanical stimulation. Contraction of the posterior edge by myosin II might therefore push the anterior portion forward, as Uchida and colleagues (Uchida et al., 2003) suggested. After this contraction and elongation, a new cycle could then begin with elongation of pseudopodia through actin polymerization (Fig. 9A).

**Fig. 7.** Traction stresses under retracting pseudopodia mediated by myosin II. (A) Simultaneous recording of GFP-myosin-II fluorescence and traction stresses using a combination of TIRF and force microscopy: the dynamics of myosin II filaments (upper images) and traction stresses (lower images) under a pseudopod of a migrating myosin-II-null cell are shown. The stress map was made from three migrating cells randomly selected from nine cells. The time elapsed is indicated in each GFP-myosin-II image. Myosin II filaments begin to accumulate in the pseudopod during elongation (0-40 seconds), followed by increased traction stress in the silicone substratum just under the pseudopod (40-80 seconds). Retraction began at the same time as the beginning of the increase in traction stress (40 seconds). Bar, 1 \( \mu m \). (B) Time-courses of pseudopod area (blue) and traction intensity of GFP-myosin-II (red). (C) Time-courses of fluorescence intensity of GFP-myosin-II (red) and average traction stress in the pseudopod (blue). The increase in pseudopod area was followed by accumulation of myosin II (B). Traction stress in the pseudopod subsequently reached a maximum during retraction (C).
cell culture

Dicytostelium discoideum cells were cultured in HL5 medium [1.3% (w/v) bacteriological peptone, 0.75% (w/v) yeast extract, 3.5 mM Na2HPO4·12H2O, 3.5 mM KH2PO4, pH 6.4] and developed until they became aggregation competent in BSS (10 mM NaCl, 10 mM KCl, 3 mM CaCl₂, pH 6.3). The cell lines used included AX2 cells (referred to as wild-type cells or HS1 expressing GFP-ABD120k, GFP-myosin-II, or GFP-E476K-myosin-II. ABD120k is the actin-binding domain of the actin-crosslinking protein ABP120 (Pang et al., 1998) and average binding stress in the pseudopod (blue). The increase in pseudopod area was followed by the accumulation of E476K myosin II in the pseudopod, as was the case for normal myosin II (Fig. 7B). However, the traction stress increased only at the tip of the retracting pseudopod.

Materials and Methods

Cell culture

Dicytostelium discoideum cells were cultured in HL5 medium [1.3% (w/v) bacteriological peptone, 0.75% (w/v) yeast extract, 3.5 mM Na2HPO4·12H2O, 3.5 mM KH2PO4, pH 6.4] and developed until they became aggregation competent in BSS (10 mM NaCl, 10 mM KCl, 3 mM CaCl₂, pH 6.3). The cell lines used included AX2 cells (referred to as wild-type cells but actually an axenic derivative of the wild-type strain NC4) expressing GFP-ABD120k, GFP-myosin-II, or GFP-E476K-myosin-II. ABD120k is the actin-binding domain of the actin-crosslinking protein ABP120 (Pang et al., 1998) and can only bind to filamentous actin. Therefore, fluorescence imaging of GFP-ABD120k reflects the distribution of filamentous actin. The gene encoding GFP-ABD120k was kindly provided by D. A. Knecht (University of Connecticut, CT). This fusion gene was inserted into the pBlG expression vector by T. Q. Uyeda (AIST). E476K mutant myosin II cannot hydrolyze ATP in myosin heavy-chain-null cells (Ruppel and Spudich, 1996; Yumura and Uyeda, 1997).

Preparation of elastic substrata

Elastic substrata were made from two kinds of materials: gelatin and silicone. Gelatin substrata were made as described previously (Doyle and Lee, 2002; Doyle and Lee, 2004) but with several modifications. Briefly, stock gels were made with 0.4% gelatin (90-110 Bloom), G6144-100G, Sigma) dissolved in BSS containing 3 mM Mes, pH 6.3 with KOH. Stock gels were liquefied at 30–40°C, and 200 μl were transferred into a round chamber (16 mm in diameter and 2 mm in depth) whose bottom was made of a 22×22 mm coverslip (No. 1, Matsunami, Japan). After solidification, fluorescent orange microspheres (200 nm in diameter, F-8809, Molecular Probes) were attached to the surface of the substrata. The subsequent procedure was identical to that described previously (Doyle and Lee, 2002; Doyle and Lee, 2004).

Silicone substrata were made according to Iwadate and Yumura (Iwadate and Yumura, 2008). Briefly, two types of platinum-catalyzed silicone (CY52-276A and B, Dow Corning Toray, Tokyo, Japan) were used. A 300 mg aliquot of CY52-276A and a 250 mg aliquot of CY52-276B were mixed. The mixture was spread on a 22×22 mm coverslip (No. 1, Matsunami, Japan). After solidification of the mixture, a round chamber (16 mm in diameter and 2 mm in depth) was assembled using the coated coverslip as the bottom of the chamber. The solidified substrata were kept in a hermetically sealed case with a 50 μl aliquot of the liquid silane (3-aminopropyl triethoxysilane, Sigma Aldrich, Tokyo, Japan) at 70°C for 1 hour in order to attach the silane to the surface of the silicone substrata by vapor deposition. Red-fluorescent carboxylate-modified microspheres (20 nm in diameter, F-8786, Molecular Probes) were attached to the surface of the substrata by means of the binding between the amino group in the silane and the carboxyl group in the microspheres. The Young’s moduli of the elastic substrata were measured using the method of Lo and colleagues (Lo et al., 2000). Briefly, a steel ball (0.5 mm diameter, 7.8 kg/m³) was placed on a substratum embedded with fluorescent beads. The Young’s modulus was calculated as

\[ Y = 3(1-p^2)2f^2/4d^{3/2}r^{1/2} \]

where \( f \) is the force exerted on the sheet, \( r \) is the radius of the steel ball, and \( p \) is the Poisson ratio [assumed to be 0.5 for gelatin (Doyle et al., 2004) and silicone (Dembo et al., 1996)]. Typical values of the Young’s modulus for gelatin and silicone substrata were 2.8 kPa and 1.0 kPa, respectively.
Microscopy

Migrating cells plated on silicone substrata were observed using a confocal microscope (Zeiss, LSM500, Germany) with a 100× objective lens (PlanFluar 100×). An argon ion laser was used at an excitation wavelength of 488 nm. GFP fluorescence was detected with a 505-530 nm band-pass filter, and the orange-fluorescent beads were detected with a 560 nm high-pass filter.

Migrating cells plated on silicone substrata were observed by a TIRF microscope assembled on an inverted microscope (Olympus, IX71, Japan) with a 60× objective lens (Lapton 60×, OTIFRM). A laser diode (DPBL-9010F, Photop Technologies, China) at a wavelength of 473 nm and a He-Ne laser (Research Electro Optics) at a wavelength of 453 nm were used for excitation of GFP and red beads, respectively. Exiting light from each laser was applied in turn to electric shutters (Copal, No. 0, Japan) in front of the laser heads. The fluorescence of GFP and red beads was detected with a dual band-pass filter (XF3056, Chroma Technology). Fluorescence images were collected using a cooled CCD camera (ORCA ER, Hamamatsu Photonics, Japan). The electromagnetic shuttering and CCD were controlled using a custom program developed under LabVIEW 7.1 (National Instruments, Japan).

Calculation of traction stresses (I)

Traction forces were not calculated as the real values of the ‘forces’ in cells on the Newton-scale but rather as ‘stresses’ in the substratum with the Pascal-scale using its three nodes. The stresses of each element were calculated from the strains of the element under the assumption that the substrata are in a plane stress state (see above or Iwadate and Yumura (Iwadate and Yumura, 2008) for further details). Briefly, migrating cells cause strains in the elastic substrate and displacements of the beads on the substratum (Fig. 1A). A representative sample image of the beads is shown in Fig. 1B. The white line indicates the outline of the migrating cell on the substratum. An image of beads comprising 512×512 pixels was divided into approximately 8000 triangle elements as shown in Fig. 1C. The length of the sides of each triangle is ~400 nm. Displacements of the nodes of each element were calculated from the displacements of the beads. The strain of each element was calculated from the displacement of its three nodes. The stresses of each element were calculated from the strains of the element under the assumption that the substrata are in a plane stress state (see below for further details).

The magnitudes of the stresses at each element under a cell were visualized by pseudocolor. Each node and its displacement vector are superimposed onto the pseudocolor images as white circles and bars, respectively. All of these calculations and some of the image processing were performed using a custom program developed under RealBASIC Ver. 3.5 (ASCII Solutions, Japan). Image processing was also performed using ImageJ Ver. 1.33, Image SXM Ver. 1.74 and Photoshop CS (Adobe, Japan).

Calculation of traction stresses (II)

In the normal finite element method, the size of the elements should be small and the density of the elements should be high at the area where the stress is expected to be large, while the size should be large and the density should be low at the area where the stress is expected to be small for accurate and fast calculations. However, the distribution of the stresses exerted by the force of a migrating cell cannot be predicted. Thus, in this study, to calculate the traction stresses as accurately as possible, a mesh in which very small triangle elements were distributed uniformly was prepared for the following calculations (Fig. 1C). The length of the sides of each element was ~400 nm (~8 pixels).

As shown in Fig. 10A, each triangle element has three nodes, A1, A2 and A3. The original positions and the displacements of the three nodes are A1 (x1, y1), A2 (x2, y2), A3 (x3, y3) and (u1, v1), (u2, v2), (u3, v3), respectively. The displacement vector, (u, v), of the arbitrary point P, whose original position is (x, y), in the triangle element is determined as:

$$
\begin{pmatrix}
  u \\
  v 
\end{pmatrix} = \mathbf{N} \mathbf{U}_n.
$$

(1)

The magnitudes of the stresses at each element under a cell were visualized by pseudocolor. The elements outside the cell were filled with black even if the stress there was not zero.

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