Dynamics of novel feet of *Dictyostelium* cells during migration

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Summary

We observed the dynamics of actin foci in live *Dictyostelium* cells expressing GFP-actin. Actin foci were dynamic structures, but they were fixed on the substratum during cell migration. Interference reflection microscopy revealed that the ventral cell membrane was closer to the substratum at sites of actin foci. Furthermore, some actin foci were incorporated into the retraction fibers, ripped off from the cells and eventually shed on the substratum after the cells moved away. The velocity of the cells was inversely proportional to the number of actin foci. Measurement of traction force using a silicone substratum demonstrated that the traction force was transmitted to the substratum through actin foci. Taken together, several lines of evidence strongly suggest that actin foci function as the active 'feet' of *Dictyostelium* cells. We also found evidence suggesting that changing step is regulated in a coordinated manner during cell migration. Possible mechanisms by which these cells migrate across substrata are discussed in this context.

Movies available on-line

Key words: Actin foci, Cell movement, Cell-substratum adhesion, Myosin, GFP

Introduction

Cell migration plays a critical role in a variety of physiological processes including growth, development and wound healing. Regulation of the attachment of cells to the substratum is essential for cell migration. The mechanisms underlying cell-substratum adhesion have been investigated primarily in fibroblasts, which are slow-moving cells with an irregular shape. Interference reflection microscopy (IRM) has shown that many focal contacts are distributed on the ventral surface of fibroblasts (Curtis, 1964; Izzard and Lochner, 1976). Focal contacts are composed of clusters of integrin, a transmembrane glycoprotein involved in cell-substratum adhesion, which are linked to the terminals of stress fibers through a series of linkage proteins, such as talin, vinculin, alpha-actinin and various signaling proteins (Burridge et al., 1988; Zamir and Geiger, 2001). The actin filaments transmit traction force to the substratum at the sites of cell-substratum adhesion (Beningo et al., 2001; Burton et al., 1999; Munevar et al., 2001; Oliver et al., 1999; Perham and Wang, 1999).

During cell migration, focal contacts form in extending lamellipodia (Izzard and Lochner, 1980). These contacts become fixed on the substratum until they reach the posterior edge of the cell (Schmidt et al., 1993; Ballestrem et al., 2001). In skeletal muscle fibroblasts and Chinese hamster ovary cells, when the adhesion structures reach the posterior edge, the tail of the cell detaches from the substratum through the breakdown of the linkage between the integrin clusters and actin filaments (Regen and Horwitz, 1992; Schmidt et al., 1993; Huttenlocher et al., 1997; Palecek et al., 1996; Shiraha et al., 1999). The integrin clusters from these cells are then shed on the substratum (Regen and Horwitz, 1992; Palecek et al.,

1998). In contrast, in 3T3 cells, the linkages between integrin clusters and actin filaments do not separate even after the focal contacts reach the posterior edge, but they instead slide inward (Smilenov et al., 1999; Ballestrem et al., 2001).

In keratocytes, which are fast moving cells with a consistent shape, dot-like contacts are distributed behind the leading edge of the lamellipodium. At these dot-like contacts, integrin clusters are linked to the cortical actin meshwork through a similar series of linkage proteins to those in fibroblasts (Lee and Jacobson, 1997). The traction force of keratocytes is generated by the interaction of actin-myosin at the base of the lamellipodium, and the force is transmitted to the substratum through the dot-like contacts described above (Burton et al., 1999; Anderson and Cross, 2000). These contacts are formed at the base of the leading edge and become fixed on the substratum as the cell migrates (Anderson and Cross, 2000).

Polymorphonuclear leukocytes are fast-moving, irregularly shaped cells. IRM analysis has shown that the cell-substratum gap is relatively uniform throughout the cell body (Keller et al., 1983). However, because integrin, talin (Lawson and Maxfield, 1995; Pierini et al., 2000), vinculin (Takubo and Tatsumi, 1999), alpha-actinin (Pierini et al., 2000) and F-actin are concentrated in the leading lamella, adhesion complexes are considered to be located at the lamella. These adhesion complexes are continuously formed at the leading edge and disassembled behind the leading lamella (Lawson and Maxfield, 1995; Pierini et al., 2000). Integrins are internalized by endocytosis at the base of the leading lamella and delivered to the leading edge for recycling (Lawson and Maxfield, 1995; Pierini et al., 2000). In the tail region, integrins are detached

from the substratum by anti-adhesive molecules (Seveau et al., 2000).

Dictyostelium cells are similar to leukocytes, in that they are fast-moving cells with an irregular shape (Murray et al., 1992; Wessels et al., 1994). Talin (Kreitmeier et al., 1995) and alpha-actinin (Brier et al., 1983) have been identified as adhesion-related proteins and their subcellular localization is similar to that of actin. IRM and total internal reflection aqueous fluorescence (TIRAF) measurements have shown that the cell-substratum gap is relatively uniform in Dictyostelium cells (Gingell, 1981; Gingell and Vince, 1982; Gingell et al., 1982; Todd et al., 1988). Two actin-containing structures have been proposed to act as 'feet' in Dictyostelium cells. One of the candidates is eupodium, but this appears only in the cells that are under the pressure of the agar sheet (Fukui and Inoue, 1997; Fukui et al., 1999). The other is actin foci, which are observed on the ventral membrane of freely migrating cells (Yumura and Kitanishi-Yumura, 1990). When the cells are stained with TRITC-ConA, distinct patterns of dots and short fibers, which are referred to as cellular tracks (CTs), are observed behind the cells (Uchida and Yumura, 1999). Since the dots in CTs contain actin and alpha-actinin, it is conceivable that they are derived from actin foci.

In Dictyostelium, for which powerful tools for molecular genetic approach can be utilized, we can clarify the mechanism of cell feet and migration by both physiological and molecular genetical approaches. In the present study, we observed the dynamics of actin foci, the most likely candidates for Dictyostelium feet, in live cells expressing actin fused to green fluorescent protein (GFP-actin). Actin foci were fixed on the substratum and remained as dots in CTs as the cells migrated. Simultaneous observations by IRM and confocal fluorescence microscopy revealed that the ventral cell membrane is closest to the substratum at actin foci. Furthermore, actin foci were the sites where the traction force of the cell was transmitted to the substratum. These findings represent the first convincing evidence that actin foci serve as the feet of Dictyostelium cells. Based on our observations of the dynamics of actin foci, we discuss possible mechanism of cell migration on the substratum, with respect to the feet acting as the scaffold to transmit force to the substratum.

Materials and Methods

Plasmid transformation
The green fluorescent protein (GFP) gene was fused to the N terminus of the Dictyostelium actin gene, which was kindly provided from Dr Knecht at the University of Connecticut. The sequence of the linker was 5′-GGTCCAGGTAAGGATCCA-3′. This fused gene was inserted into the pBIG expression vector, which contains a G418 resistance cassette (Ruppel et al., 1994). The resulting plasmids were transformed into wild-type Dictyostelium cells (strain AX2) and myosin heavy chain null (HS1) Dictyostelium cells by electroporation (Yumura et al., 1995). Transformed cells were selected in HL-5 medium [1.3% (w/v) bacteriological peptone, 0.75% (w/v) yeast extract, 85.5 mM D-glucose, 3.5 mM Na$_2$HPO$_4$·12H$_2$O, 3.5 mM KH$_2$PO$_4$, pH 6.4] in the presence of 10 µg/ml G418, as described previously (Yumura and Uyeda, 1997).

Cell culture
AX2 and HS1 cells were cultured in plastic plates containing 10 ml of HL-5 medium at 22°C. The cells expressing GFP-actin were grown in HL5 medium supplemented with G418 (10 µg/ml). Cells were harvested during the logarithmic growth phase, suspended in BSS (10 mM NaCl, 10 mM KCl, 3 mM CaCl$_2$ and 2 mM MOPS, pH 6.4), spread on non-nutrient agar and incubated at 22°C for 4 to 6 hours.

Microscopy and acquisition of images of live cells
Well-cleaned coverslips (18×18 mm; Matsunami Glass, Japan) were incubated with 500 mg/ml bovine serum albumin (BSA) for 1 hour and then vigorously washed with distilled water, unless otherwise specified. For the observation chamber, a solid silicone sheet (17×17×0.5 mm) with a square hole of about 10×10 mm was attached to the coverslip. The chambered coverslip was filled with cells suspended in BSS and a glass slide was placed over the chamber to seal the fluid. An inverted microscope (Axiovert 135M; Carl Zeiss, Germany) equipped with a confocal system (LSM 510; Carl Zeiss) was used primarily with a 100× plan neofluar oil objective lens. In order to selectively observe the ventral surface of the cells, optical sections were set at a thickness of 1.0 µm. For simultaneous observation by confocal fluorescence microscopy and interference reflection microscopy (IRM), wavelengths of 488 nm (Argon laser, 0.1% of 15 mW) and 543 nm (HeNe laser, 10% of 1 mW) were selected. The excitation beam was reflected by a beam splitter HFT488/543 (Zeiss). This beam splitter transmitted 7% of the reflections. The fluorescence emission and the reflections were separated by a beam splitter NFT545 (Zeiss) and then filtered by BP505-530 (Zeiss) and LP530 (Zeiss), respectively. Sequential images (512×512 pixels, 12 bits) were stored in a computer, typically at intervals of 2.5 seconds and analyzed with Scion Image software (Scion Corporation, Frederick, MD). All observations were performed at 22°C.

Fluorescence microscopy of fixed cells
Cells suspended in BSS were settled on clean coverslips in a moist chamber at room temperature. Cells were allowed to migrate for several hours. The coverslips were then fixed in ethanol containing 1% (w/v) formaldehyde at –17°C for 5 minutes. After washing with phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 80 mM Na$_2$HPO$_4$, pH 7.3) for 15 minutes, the cells were stained with 0.6 mg/ml tetramethyl rhodamine isothiocyanate-concanavalin A (Sigma Chemicals) for 30 minutes at 22°C. Anti-actin immunostaining was performed as described previously (Okazaki and Yumura, 1995).

Preparation of silicone rubber film and microscopy
A silicone substratum was prepared using the method described by Uchida et al. (Uchida et al., 2003). Smaller fluorescent beads (0.1 µm in diameter, Fluoresbrite YO carboxylated microsphere, Polyscience) were used to examine in detail the traction force beneath cells.

Quantitative analysis
Gained area was measured according to the method of Uchida et al. (Uchida et al., 2003). The gained area was calculated over time by subtracting the retraction area from the extension area. The extension area was defined as the increase in area determined from cell contours taken from two sequential images and the retraction area was defined as the decrease in area between 2 sequential images (Weber et al., 1995; Yumura and Fukui, 1998).

Instantaneous velocity of cells was calculated by the method described previously (Uchida et al., 2003). Displacement of the centroid of a cell in the first and the third images of three successive images was measured and then divided by the time interval. The second image was used for examining the number of the actin foci.

To analyze the traction force using the flexible silicone substratum, the position of beads in an image was measured at the center of the beads. If movement of a bead was less than 0.2 µm, the bead movement was not quantified. Image processing was performed by macro programs in Scion image software (Scion Corp.).

Results

Actin foci are shed on the substratum as the cells migrate

In our previous report, when *Dictyostelium* cells were allowed to migrate on a coverslip and then stained with Concanavalin A (Con A), tracks with distinct patterns of dots and short fibers were observed behind the cells (Uchida and Yumura, 1999). We designated these tracks as 'cellular tracks', or CTs. We hypothesized that CTs originally served as the feet of these cells and that they were shed on the substratum during cell migration. Furthermore, multiple actin foci are found on the ventral membrane of *Dictyostelium* cells and these have also been proposed to act as feet (Yumura and Kitanishi-Yumura, 1990). To examine the correlation between actin foci and dots in CTs, migrating cells were fixed on a coverslip and double-stained with TRITC-Con A and anti-actin antibodies. Fig. 1A shows the cellular distribution of actin, which was mainly localized at anterior extensions, as previously reported (Yumura et al., 1984). Several actin foci were observed on the ventral cell membrane and small fluorescent dots were observed behind the cell (Fig. 1C). Fig. 1B shows a confocal fluorescence image of the distribution of Con A receptors on the ventral cell surface. Most of the fluorescent dots were observed not only in the CTs (Fig. 1D), but also in the cell body (arrowheads in Fig. 1B). In addition, membranous staining was observed on the cell membrane, Golgi apparatus and endoplasmic reticulum. Interestingly, most of the actin foci were co-localized with the fluorescent dots, as revealed by TRITC-Con A staining (Fig. 1B,D). Therefore, it is conceivable that the dots in CTs are derived from actin foci and that these remain on the substratum as cells move.

Dynamics of actin foci in live cells

In order to observe the dynamics of actin foci in living cells, wild-type *Dictyostelium* cells were transformed with an extrachromosomal vector harboring a GFP-actin fusion gene. Constitutive expression of the gene was driven by the *Dictyostelium* 15 actin promoter. Fig. 2 shows the simultaneous observation of live GFP-actin cells by fluorescence and interference reflection microscopy (IRM). Time-lapse images of GFP-actin expression in a quiescent cell (Fig. 2A-H) showed that several fluorescent dots were present on the ventral cell membrane. The diameter of the fluorescent dots ranged from 0.3 to 1.0 µm, with an average diameter of 0.53 ± 0.12 µm (s.d., n=30), which is consistent with the size of actin foci in fixed cells. In addition, when the GFP-actin cells were fixed and stained with Con A and anti-actin antibodies, GFP-actin dots corresponded to the actin foci stained with ConA and anti-actin (data not shown). Interestingly, in live cells, the appearance of actin foci was transient and their positions on the substratum were unchanged.

In IRM images, which show the distance between the cell surface and the substratum, the gray areas show a separation
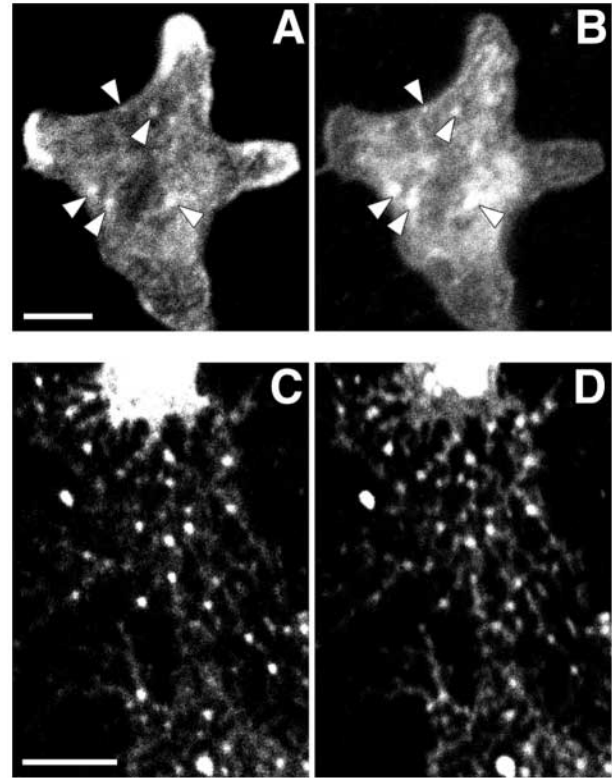

Fig. 1. Distribution of actin and ConA receptors on the ventral membrane of cells. Cells were fixed and stained with TRITC-Con A, and immunostained with anti-actin antibodies. (A,C) Distribution of actin. Several actin foci were observed on the ventral membrane of the cell (A) and small dots were observed behind the cell (C). (B,D) Distribution of Con A receptors. Fluorescent dots (B) and CTs (D) were observed on the ventral cell membrane and behind the cell, respectively. Note that the fluorescent dots of ConA receptors were co-distributed with actin foci (see arrowheads in A and B). The dots in CTs and the actin dots behind the cell were also co-distributed (C,D). The cells in upper panels and lower panels are different. Scale bar: 5 µm.

of about 30 nm and white areas show a separation of 100 nm or more (Izzard and Lochner, 1976). The frequent appearance of moving black areas was due to cytoplasmic vesicles, which come close to the cell membrane (Gingell et al., 1982). In time-lapse images of IRM observations (Fig. 2A′-H′), the tone of most of the ventral surface was gray, except for the cell margin. In careful observation of both GFP-actin and IRM images, we noticed the appearance of white rings surrounding the actin foci in IRM images (1, 2 and 3 in Fig. 2). Fig. 2I shows enlarged images of the individual actin foci and the white rings labeled as 1, 2 and 3 in Fig. 2B,E and H; the appearance of the actin foci and the white rings are almost simultaneous. In addition, the tone of the areas just corresponding to actin foci was slightly darker than the other areas of the ventral surface, indicating that the cell surface is closer to the substratum at the actin foci than most other parts of the ventral surface (Fig. 2J).

The fluorescence intensity of actin foci labeled as 1, 2 and 3 in Fig. 2B, E and H was plotted over time (Fig. 3A). This plot clearly demonstrates that the actin foci were temporary structures. Initially, actin foci were small, then grew in size and eventually shrank. After shrinkage, the
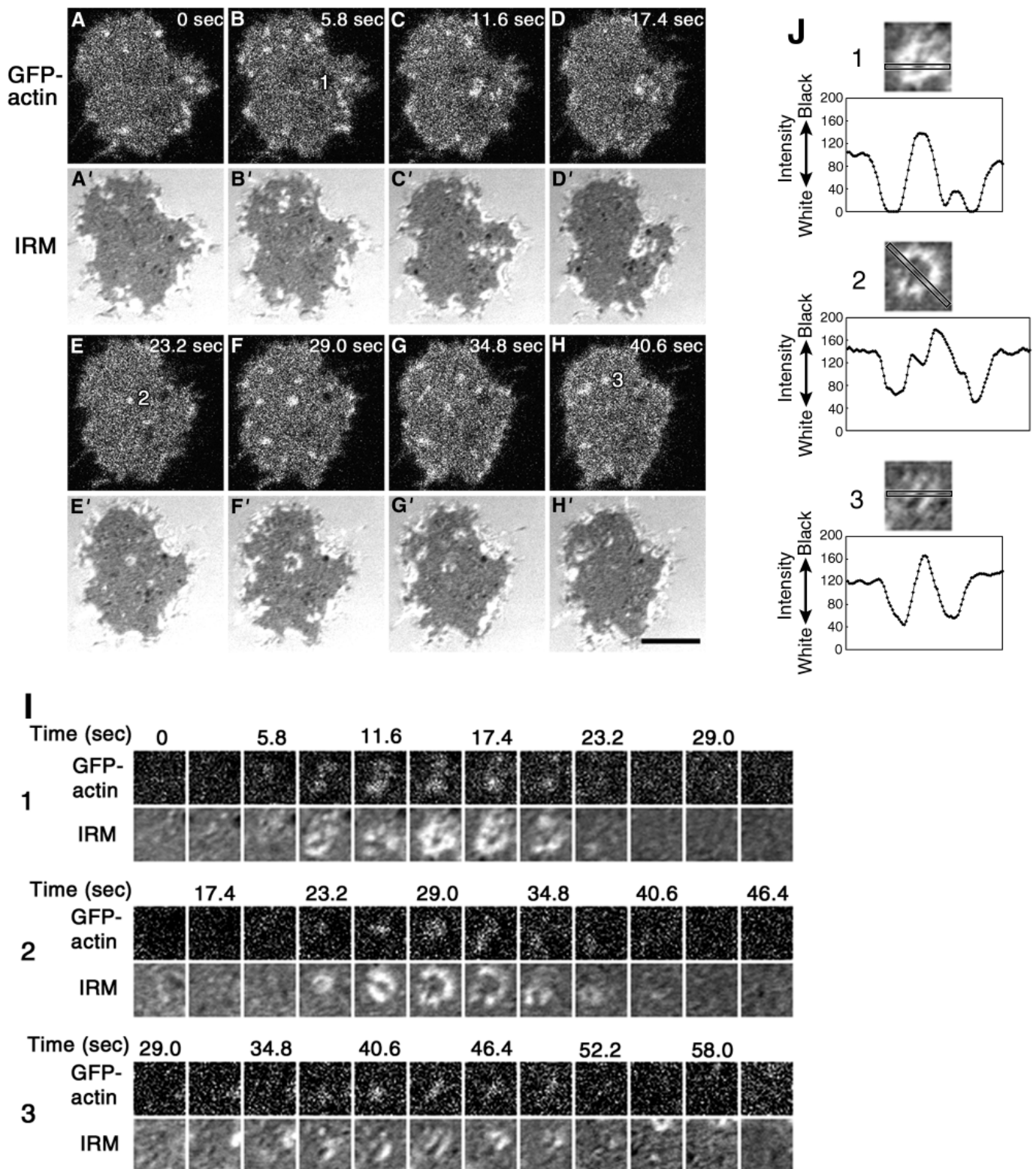
Fig. 2. Dynamics of actin foci in live cells as seem by interference reflection microscopy (IRM). (A-H) The ventral membrane of a quiescent *Dictyostelium* cell was analyzed at various time points, as indicated. (A′-H′) The distance between the cell and the substratum is indicated as tone in IRM images. While most of the ventral membrane was gray, white rings were observed surrounding actin foci (1, 2 and 3). Scale bar: 5 μm. (I) Enlarged images of the actin foci numbered 1, 2 and 3 in A-H. (J) Intensity of the tone in the area enclosed by the bars across the white ring at the actin foci numbered 1, 2 and 3. The tone at the actin foci is darker than other areas, demonstrating that the actin foci were closer to the substratum than the rest of the ventral membrane. A movie is available (Movie 1, http://jcs.biologists.org/supplemental/).
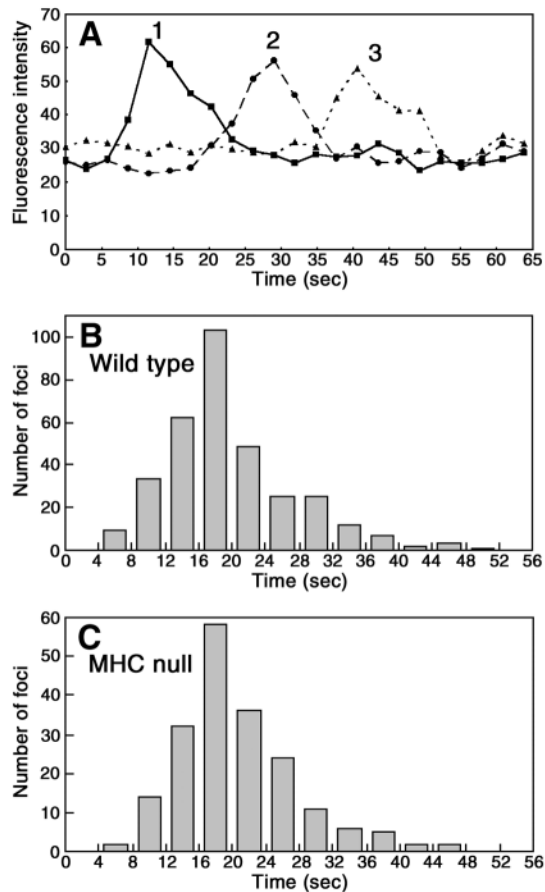
Fig. 3. Actin foci disappeared about 20 seconds after their appearance. (A) Time course of fluorescence intensity of the three actin foci shown in Fig. 2I. (B) Frequency distribution of the duration of actin foci in wild-type cells. The average±s.d. from 164 foci in 24 cells was 19.43±8.16 seconds. (C) Frequency distribution of the duration of actin foci in MHC-null cells. The average±s.d. from 192 foci in 14 cells was 20.26±7.39 seconds.

fluorescence returned to baseline or undetectable levels. Fig. 3B shows a histogram of the duration of these actin foci. The average duration was 19.43±8.16 seconds (based on 164 foci in 24 cells), suggesting that actin foci are highly dynamic structures.

Some actin foci are shed on substratum as cell migrates

In order to confirm whether actin foci were shed on the substratum as dots of CTs, the dynamics of actin foci were observed during cell migration (Fig. 4). Some actin foci disappeared, but others, especially those located in the rear of the cells, were incorporated into retraction fibers, ripped off from the cells and left as dots within CTs on the substratum (Fig. 4, arrowheads).

Interestingly, the sites from which actin foci had disappeared were also incorporated into retraction fibers, ripped off from the cell and left as dots within CTs (Fig. 4, circles), suggesting that these sites remained attached to the substratum even after the actin foci disappeared. Fig. 4M shows superimposed images of the posterior region of the migrating cell shown in

A-L, which reveals the history of retraction fibers and positions of the actin foci (shown as black dots in the figure). Most of the actin foci were located within the tracks of retraction fibers. These observations suggest that adhesion persists even after the actin foci disappear.

Velocity of migrating cells decreases as the number of actin foci increases

If actin foci serve as cell-substratum adhesion sites, cells harboring more actin foci would be predicted to move more slowly. Fig. 5 shows the relationship between the number of actin foci and the instantaneous velocity of the cell. The instantaneous velocity was calculated as the distance between the centroids of two sequential images of a cell. The velocity of the cells was inversely proportional to the number of actin foci.

The number of actin foci in myosin II heavy chain (MHC) null cells was much greater than that of wild-type cells. The average number of actin foci in wild-type cells was 6.7±3.9 (*n*=99), whereas MHC null cells exhibited an average of 19.7±11.2 (*n*=106) actin foci. This may explain, at least in part, why MHC null cells move much more slowly than wild-type cells (Wessels et al., 1988; Yumura and Uyeda, 1997). The longer duration of actin foci than in wild-type cells may increase the number of actin foci in MHC null cells and this may influence the cell velocity. But, the average duration of actin foci in MHC null cell was 20.26±7.39 seconds (192 foci of 14 cells), which is similar to that of wild-type cells (Fig. 3B,C). These results suggest that myosin II can be involved in the regulation of the number of actin foci without changing their duration.

Actin foci transmit traction force to the substratum

Traction force may be transmitted to the substratum through adhesion sites. To examine whether actin foci transmit traction force, the distribution of actin foci and traction force were simultaneously observed, using fluorescent beads attached to a flexible silicone substratum. Fig. 6A-C shows the movement of beads underneath a representative migrating cell. The traction force caused deformation of the substratum and movement of the beads. Fig. 6D shows the time-lapse images of the movement of beads in the areas numbered 1, 2 and 3 in Fig. 6B and C. In most cases, significant movement was observed only in areas surrounding actin foci. These findings strongly suggest that the traction force is transmitted through the actin foci themselves.

Coordinated changing steps during cell migration

Given that actin foci are the most probable feet of *Dictyostelium* cells, it is important to examine how the cell changes step during migration. During migration, *Dictyostelium* cells alternate between the extension and retraction phases (Uchida et al., 2003). We examined the role of actin foci in this cyclic phase change during cell migration. Fig. 7A-L and A′-L′ show time-lapse images of the distribution of actin foci in cells during migration. Fig. 7M and M′ show cyclic changes in the gained area of the cells in Fig. 7A-L and A′-L′, respectively. Contours of a cell taken at two sequential time points were superimposed and the difference between
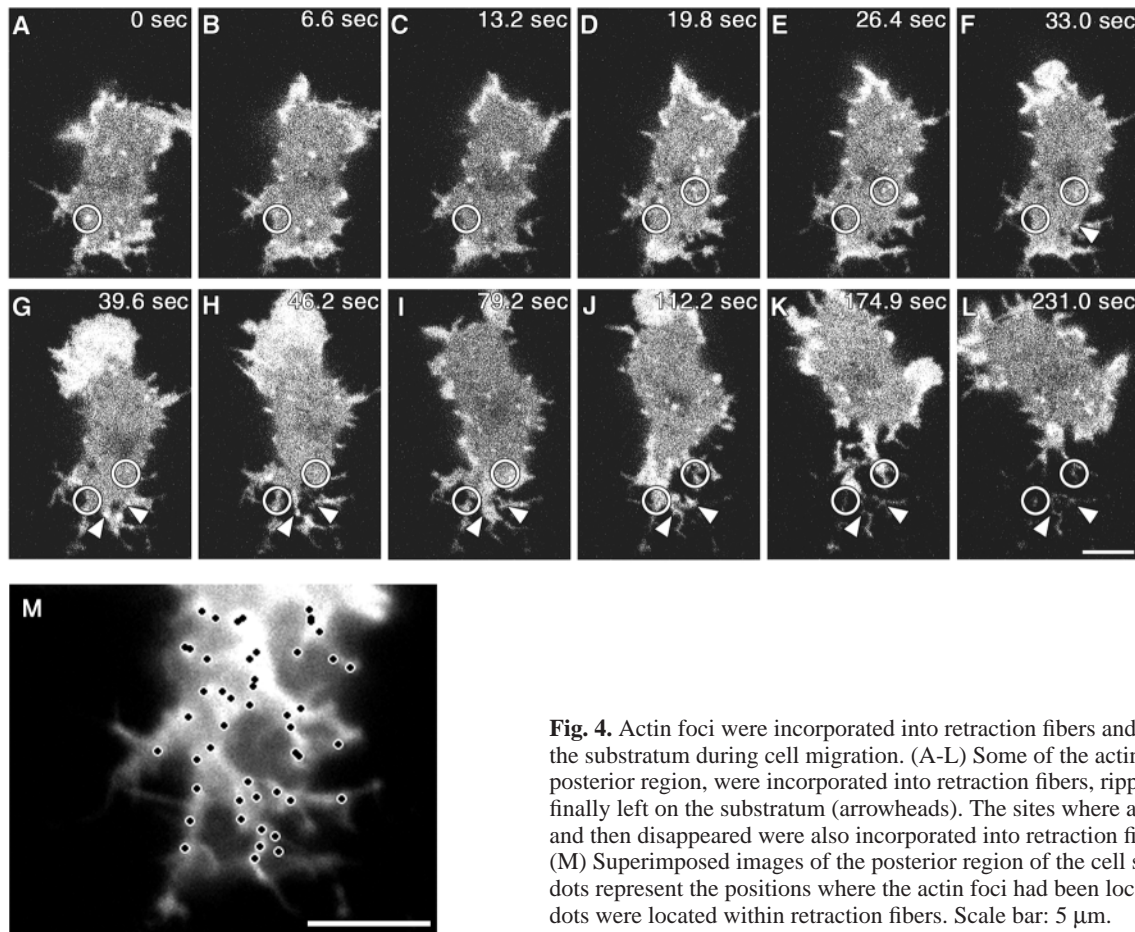
Fig. 4. Actin foci were incorporated into retraction fibers and were eventually shed on the substratum during cell migration. (A-L) Some of the actin foci, especially in the posterior region, were incorporated into retraction fibers, ripped off from the cell and finally left on the substratum (arrowheads). The sites where actin foci had appeared and then disappeared were also incorporated into retraction fibers (circles).
(M) Superimposed images of the posterior region of the cell shown in A-L. The black dots represent the positions where the actin foci had been located. Note that all these dots were located within retraction fibers. Scale bar: 5 μm.

extension and retraction areas (gained area) was plotted over time. If the difference is greater than zero, extension is more prominent than retraction (the extension phase). If the difference is less than zero, retraction is more prominent than extension (the retraction phase). Fig. 7M and M′ show that the cells alternate between the extension and the retraction phases.

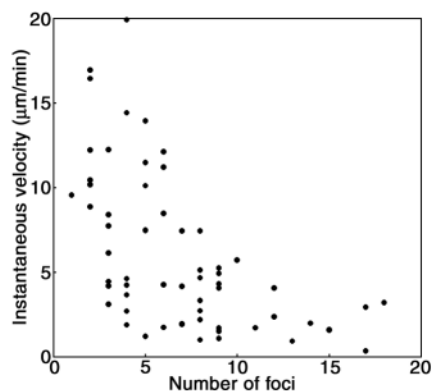The distribution and the number of actin foci were correlated

Fig. 5. The instantaneous velocity of cells was inversely proportional to the number of actin foci. The number of actin foci and instantaneous velocity were examined in sequential images of eight cells.

with the cyclic phase change of cell migration. During the early to middle extension phase, actin foci were distributed in both the anterior and the posterior regions of the cell (Fig. 7A,B,G,H,A′,B′,G′,H′ and Fig. 8). From the late extension phase to the beginning of the retraction phase, the number of actin foci in the posterior region remarkably decreased or disappeared entirely (Fig. 7C-E, I-J,C′-D′,I′-K′ and Fig. 8). At the same time, the posterior region of the cell contracted and detached from the substratum (Uchida et al., 2003), suggesting that the decreased number of actin foci could be related to the detachment and the contraction of the posterior regions of migrating cells. During the retraction phase, the number of actin foci in the posterior region increased again (Fig. 7F,L,E′-F′,L′ and Fig. 8). Taken together, these observations suggest that *Dictyostelium* cells change steps in a coordinated manner during cell migration.

Fig. 9A-M show a typical distribution of actin foci in a migrating MHC null cell. Actin foci were scattered randomly throughout the ventral cell membrane and we could not identify any consistent pattern either in the number or distribution of actin foci. Although MHC null cells also alternated between the extension and retraction phases (Fig. 9N), the number of actin foci did not decrease in the posterior region of the cells during the transition from the late extension to the early retraction phase (Fig. 9D-F,J-M). These results suggest that the decrease in the number of actin foci in the posterior region could be mediated by myosin II.
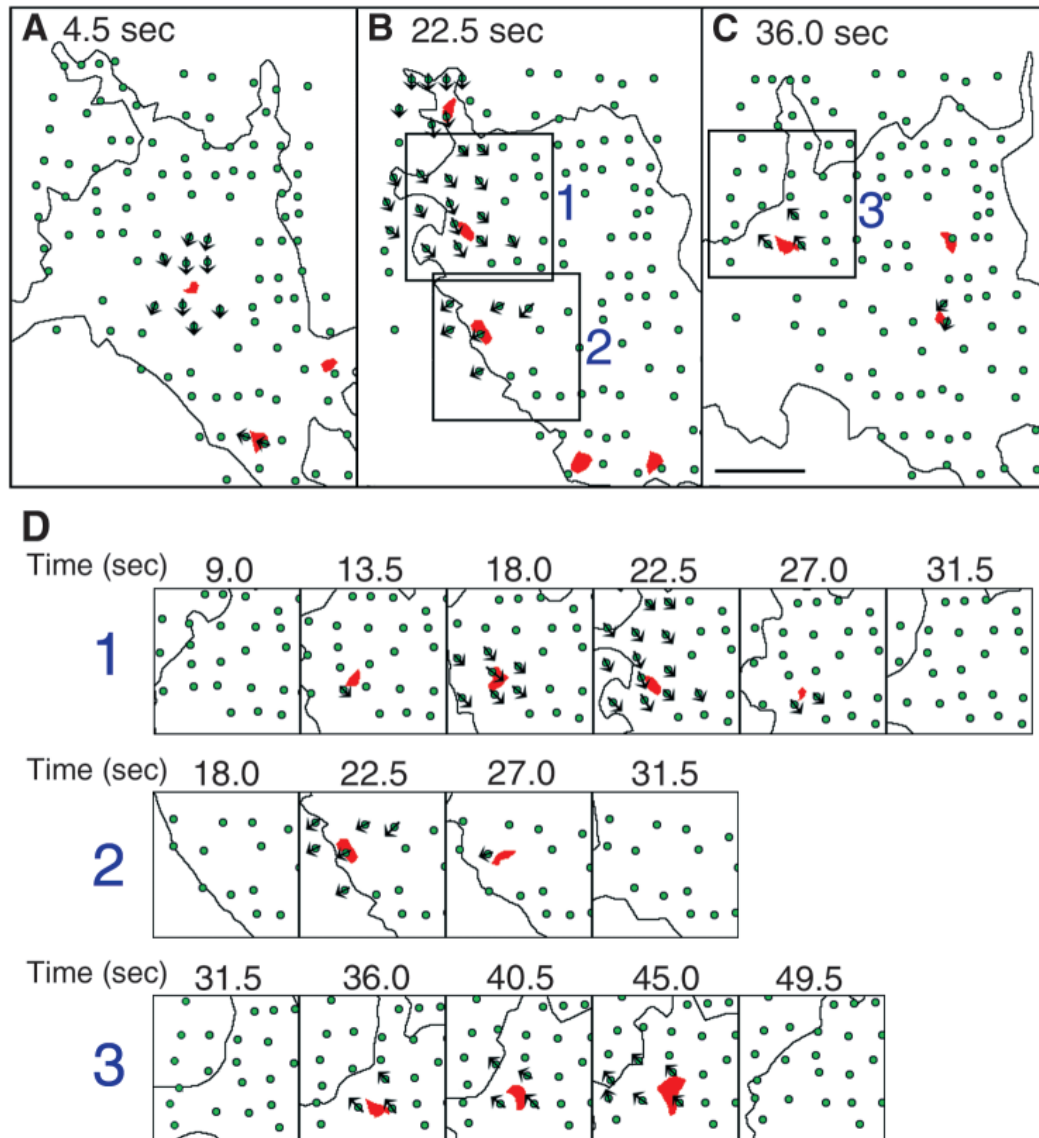
Fig. 6. Traction force was transmitted to the substratum through actin foci. (A-C) Simultaneous observations of actin foci and bead movements beneath the cell. Time 0 indicates the beginning of the observation. Red areas represent the actin foci and green dots represent the position of the beads. Arrows represent the vectors of the bead movements during the previous 4.5 seconds. The length of the arrows is three times as long as actual displacement of the beads. Interestingly, extensive movement of the beads was observed only around the actin foci. A soft silicone substratum (about 4 nN/μm in stiffness) was used in order to exaggerate the movement of the beads, as described previously (Uchida et al., 2003). Scale bar: 5 μm. (D) Time-lapse images of the areas numbered 1, 2 and 3 in B and C. Around the actin foci, extensive bead movements were observed.

Discussion

This study provides the first characterization of dynamic feet of *Dictyostelium* cells, through the observation of actin foci in live cells expressing GFP-actin. Several lines of evidence suggest that actin foci serve as the feet of *Dictyostelium* cells. First, actin foci were fixed on the substratum and some were incorporated into retraction fibers. As the cells migrated, these foci were eventually shed on the substratum and left as dots within CTs. Second, IRM analysis revealed that the cell surface was closer to the substratum in the areas of the actin foci, as compared to other areas of the ventral cell membrane. Third, the velocity of cells decreased as the number of actin foci increased. Fourth, traction forces were detected underneath

cells only in the areas surrounding actin foci. Finally, the number and the distribution of actin foci changed in coordination with the cyclic oscillation between the extension and retraction phases that occurs during migration.

What is the role of actin in actin foci? Staining with rhodamine-labeled phalloidin enabled us to visualize actin foci in fixed cells (Yumura and Kitanishi-Yumura, 1990). Electron microscopy analysis showed that numerous actin filaments emerged from electron-dense structures in the center of actin foci (Yumura and Kitanishi-Yumura, 1992). These observations indicate that actin foci are composed of a polymerized form of actin and that the formation of actin foci can be explained by polymerization of actin monomers or
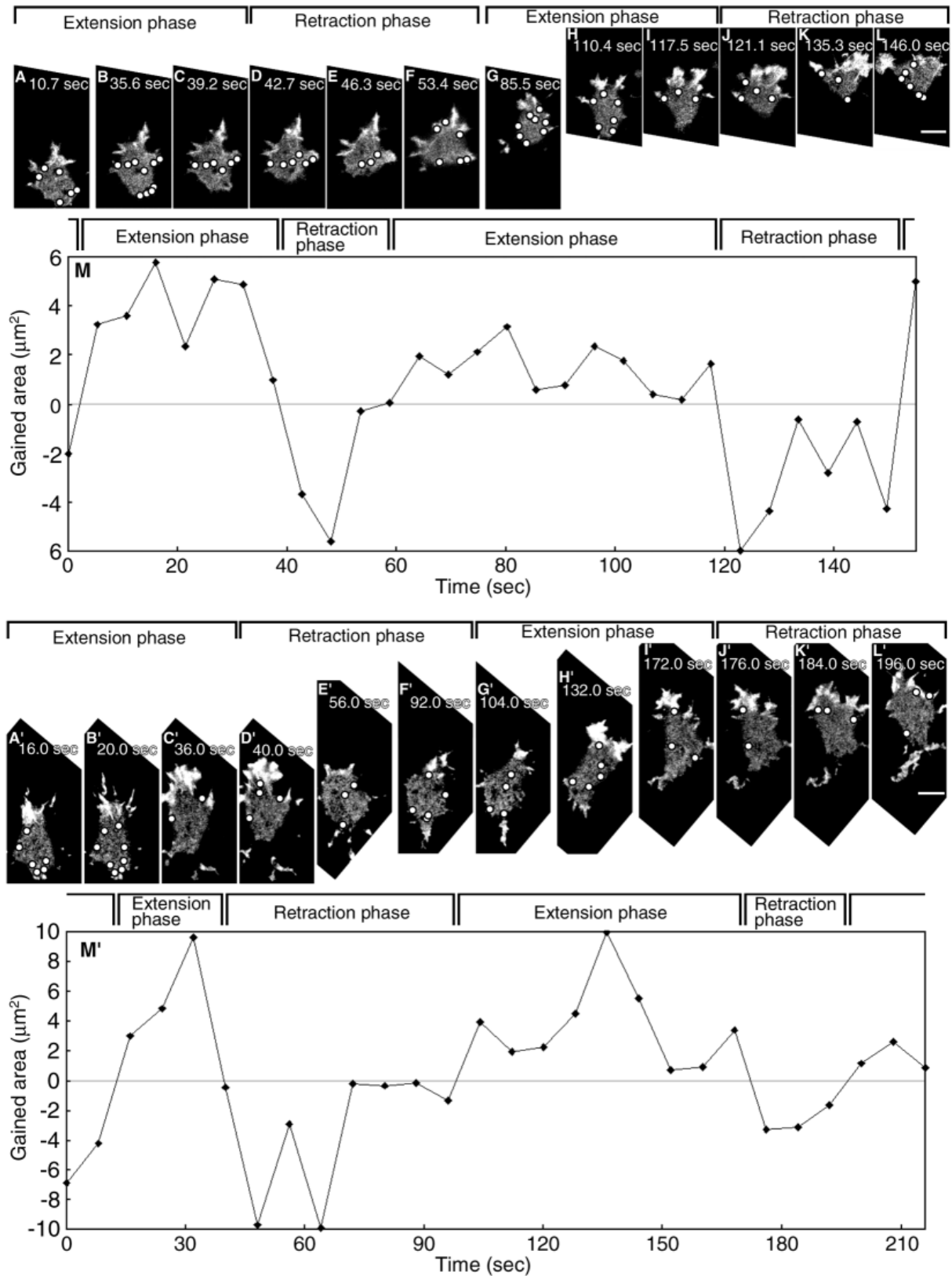
Fig. 7. The number of actin foci decreased in the posterior region during the retraction phase. Panels A-L and A′-L′ show sequential images of actin foci in two representative migrating cells. The actin foci were marked with white spots. M and M′ show the time course of the area gained by the cells shown in A-L and A′-L′. The cells alternated between the extension and the retraction phases. Note that the changes in the distribution of actin foci were correlated with the cyclic phase changes during migration. From the late extension phase to the early retraction phase, the number of actin foci in the posterior region decreased (C-E, I-J, C′-D′ and I′-K′). Scale bar: 5 μm.
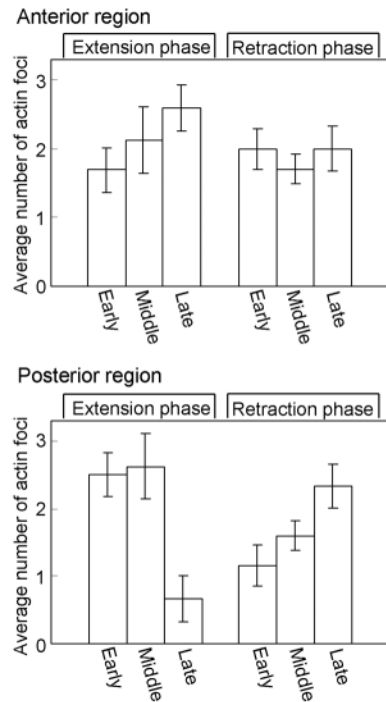
Fig. 8. Average number of actin foci in the anterior and the posterior regions during extension and retraction phases. The boundary between the anterior and the posterior regions of the cell was defined by the perpendicular line that crossed the half point of the cell migration axis between the extreme anterior and posterior edges (small protrusions were excluded). The cell migration axis was defined from the cell centroids in three successive images (Uchida and Yumura, 1999). Both extension and retraction phases were divided into early, middle and late extension (or retraction) phases respectively, and the number of the actin foci was counted. Vertical bars showed standard errors of 11 cases of six cells. In the anterior region, the number of the actin foci did not significantly fluctuate. In the posterior region, the number of the actin foci significantly decreased (*t*-test, $P<0.01$) in the late extension phase, and then gradually increased.

recruitment of preexisting actin filaments. As for the role of actin filaments in actin foci, one possibility is that actin filaments link the cell-substratum adhesion sites to the entire cytoskeletal framework or cortical actin meshwork and thereby transmit the traction force to the substratum. The findings that cytochalasin D, which depolymerizes actin, and butanedione monooxime, which inhibits myosin ATPase, relax the traction force to the substratum in mammalian cells also support this hypothesis (Pelham and Wang, 1999; Oliver et al., 1999; Riveline et al., 2001). As a second possibility, actin filaments may recruit other components necessary for cell-substratum adhesion to actin foci. After the recruitment of these components, actin filaments may not be required, because we showed that cell-substratum adhesion persisted even after actin foci disappeared. Alternatively, a ground level of actin equivalent to the cortical actin layer may still function to maintain adhesion.

IRM analysis identified the appearance of white rings around actin foci, indicating that these areas are farther from the substratum than other areas of the ventral membrane (Fig. 2).

However, the centers of the rings where actin foci were situated were closer to the substratum, as judged from their tone. Therefore, a third possible role of actin filaments within actin foci is that they may act as suction cups to mediate non-specific adhesion to the substratum. If a projection such as a microvillus extends through the polymerization of actin and pushes the substratum, the cell surface surrounding the projection moves back from the substratum to form a ring-shaped space, which may temporarily generate suction force. This could be attributed, in part, to non-specific adhesion to the substratum. These three possible roles are not mutually exclusive.

The molecular targets for adhesion to the substratum, such as collagen and fibronectin, have been extensively characterized in mammalian cells. There is no significant extracellular matrix in the case of *Dictyostelium* cells prior to the multicellular stage, but cells can adhere to the surface of coverslips and the bottom of plastic dishes. Some homologues of mammalian cell-substratum adhesion-related proteins, such as alpha actinin and talin have been reported in *Dictyostelium*. Mutant cells lacking talin, a focal adhesion protein found in mammalian cells, are defective in cell-substratum adhesion (Niewohner et al., 1997). Alpha-actinin (ABP-95), myosin IB, actin-bundling protein (ABP-30a) (Okazaki and Yumura, 1995) and gelation factor (ABP-120) have been found to be localized at actin foci and CTs (unpublished data). It has been suggested that myosin VII, phg1 and rasG may also be involved in cell-substratum adhesion (Tuxworth et al., 2001; Cornillion et al., 2000; Chen and Katz, 2000). Recently, Sad A was identified as a membranous adhesion protein in *Dictyostelium* (Fey et al., 2002). Sad A contains nine putative transmembrane domains and three conserved EGF-like repeats in its predicted extracellular domain. The EGF repeats are similar to corresponding regions in other proteins that function in adhesion, such as tenascins and integrins.

Myosin II seems to decrease the number of actin foci, because MHC null cells exhibit more actin foci than wild-type cells. Myosin II does not localize within actin foci, but the addition of ATP induces the accumulation of myosin II around these structures in membrane-cytoskeleton complexes in a semi in vivo system (Yumura and Kitanishi-Yumura, 1992). This suggests that there is some continuous linkage through which the force generated by the interaction between actin and myosin II can be transmitted to the substratum via actin foci. Myosin II is primarily localized in the posterior region of migrating cells, where it causes contraction, which may break the adhesion in the posterior region during the retraction phase. Alternatively, myosin II may participate directly in the disappearance of actin foci and the release of adhesion in the posterior region during the retraction phase. In a previous study, we examined traction force using a silicone substratum to predict myosin II-dependent release of adhesion in the posterior region during the retraction phase (Uchida et al., 2003).

How does the cell regulate the changing steps in a coordinated manner? Since MHC null cells did not show coordinated changes in steps in the posterior region, myosin II may partially mediate these functions. However, the mechanism is likely to be more complicated, because coordinated changes in step requires precise control of both timing and positioning of the cellular steps over the entire cell body. Local changes in intracellular Ca^{2+} concentrations may
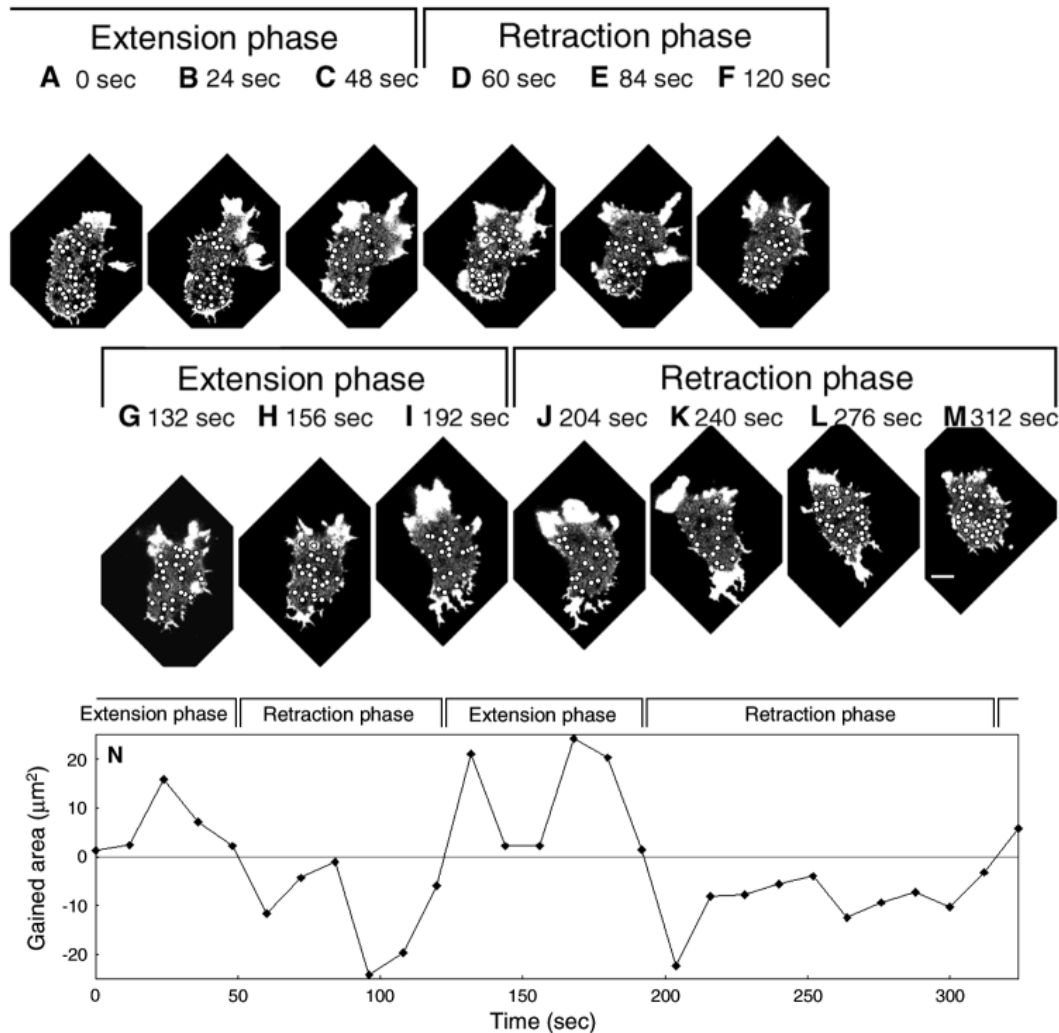
Fig. 9. Dynamics of actin foci in MHC null cells. (A-M) Sequential images of actin foci in a representative MHC null cell. Actin foci marked with white spots were consistently observed in both the anterior and the posterior regions of MHC null cells, in contrast to wild-type cells. Scale bar: 5 μm. (N) Time course of the area gained by the MHC null cell shown in A-M. The MHC null cell also alternated between the extension and the retraction phases.

regulate the coordination. This possibility is supported by the fact that the number of actin foci is transiently decreased when the cells are treated with a Ca^{2+} ionophore (Yumura, 1993; Yumura et al., 1996).

Fig. 10 shows a schematic model illustrating how cells migrate across the substratum. Our previous study of traction force using a silicone substratum demonstrated that *Dictyostelium* cells alternate between extension and retraction phases during migration. Traction force is generated as the reaction force to the motive force of the anterior extension during the extension phase. During the retraction phase, traction force in the posterior region diminishes and the posterior contraction, in turn, induces the anterior extension (Uchida et al., 2003). Findings from the current study about the feet of these cells may add valuable information to our previous model.

In the present model, we propose that actin foci link the cortical actin meshwork with putative membrane proteins that adhere to the substratum (Fig. 10Aa). Motive force for cell

migration that is generated in the cell body is transmitted to the substratum through actin foci. When actin foci appear, putative membrane proteins gather around them. These proteins remain adhered to the substratum even after actin foci have disappeared (Fig. 10Ab).

During the extension phase (Fig. 10Ba-c), the anterior region of the cell extends, which may be driven by actin polymerization or by myosin I. This extension requires scaffolds at the base of the extending anterior region. Actin foci situated at the base of the anterior extension most probably act as the scaffolding for this anterior extension. The newly extended region then becomes anchored by new actin foci formed at the forefront of the anterior region. From the end of the extension phase to the beginning of the retraction phase, the number of actin foci in the posterior region decrease or disappear entirely (Fig. 10Bd). This process is driven by myosin II, as discussed above. At the beginning of the retraction phase, the posterior region begins to contract in response to the force generated by myosin II in this region,
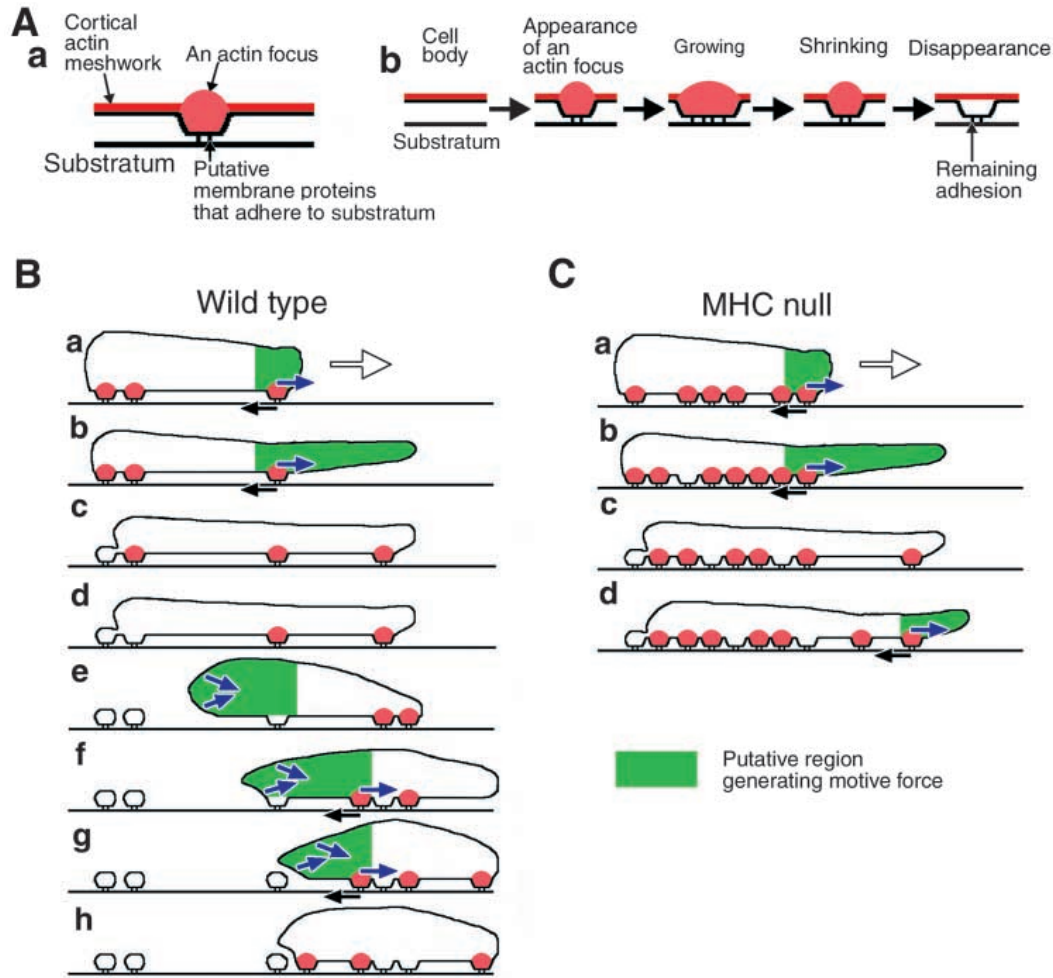
Fig. 10. A schematic model illustrating how cells migrate across a substratum with coordinated changing steps. (Aa) Putative structure of cell-substratum adhesion sites. Actin filaments (pink) in an actin focus link the cortical actin layer (red) to the substratum through putative membranous cell-substratum adhesion proteins (vertical short bars). (b) Dynamic behavior of the adhesion structures. Following the appearance of actin foci, the putative membrane proteins are recruited to form adhesion structures. These proteins remain adhered to the substratum even after disappearance of the actin foci. (B) Coordinated changing steps in a wild-type cell. An open arrow represents the direction of cell migration. Green areas represent the putative region generating motive force (blue arrows) as suggested previously (Uchida et al., 2003). During the extension phase (a-c), the cell transmits traction force (black arrows) to the substratum through actin foci, which act as scaffolding for the anterior extension. (c) After anterior extension, the forefront of the newly extended region is anchored to the substratum by newly formed actin foci. (e-h) During the retraction phase, contraction and detachment occur in the posterior region. At that time, the actin foci disappear in the posterior region. Posterior contraction then generates a pushing force for anterior extension (blue arrows in f and g). The actin foci in the anterior region during this phase act as scaffolding for the anterior extension. (C) In the case of MHC null cells, generation of motive force is limited to the anterior region. These cells exhibit defects in contraction and detachment of the posterior region

which pushes the anterior region forward (Fig. 10Be-g). Throughout this process, precise regulation of changing steps in both the anterior and posterior regions is required for efficient migration. Because MHC null cells are deficient in posterior contraction (Fig. 10C), these cells always drag their bodies by the propulsive force generated in the anterior region.

In conclusion, *Dictyostelium* cells adhere and transmit traction force to the substratum through actin foci via coordinated changing steps. Where and how the cell generates propulsive force remains to be determined. Understanding the cellular signals that mediate these coordinated changing steps will help to elucidate the molecular mechanisms underlying cell migration.

References

Anderson, K. I. and Cross, R. (2000). Contact dynamics during keratocyte motility. *Curr. Biol.* **10**, 253-260.

Ballestrem, C., Hinz, B., Imhof, B. A. and Wehrle-Haller, B. (2001). Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J. Cell Biol.* **155**, 1319-1332.

Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V. and Wang, Y. L.

(2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J. Cell Biol.* **153**, 881-888.

Brier, J., Fechheimer, M., Swanson, S. and Taylor, D. L. (1983). Abundance, relative gelation activity and distribution of the 95,000 dalton actin-binding protein from *Dictyostelium discoideum*. *J. Cell Biol.* **97**, 178-185.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Dev. Biol.* **4**, 487-525.

Burton, K., Park, J. H. and Taylor, D. L. (1999). Keratocytes generate traction forces in two phases. *Mol. Biol. Cell* **10**, 3745-3769.

Chen, C. F. and Katz, E. R. (2000). Mediation of cell-substratum adhesion by RasG in *Dictyostelium*. *J. Cell Biochem.* **79**, 139-149.

Cornillon, S., Pech, E., Benghezal, M., Ravanel, K., Gaynor, E., Letourneur, F., Bruckert, F. and Cosson, P. (2000). Phg1p is a nine-transmembrane protein superfamily member involved in *Dictyostelium* adhesion and phagocytosis. *J. Biol. Chem.* **275**, 34287-34292.

Curtis, A. S. G. (1964). The mechanism of adhesion of cell to glass: A study by interference refection microscopy. *J. Cell Biol.* **20**, 199-215.

Fey, P., Stephens, S., Titus, M. A. and Chisholm, R. L. (2002). SadA, a novel adhesion receptor in *Dictyostelium*. *J. Cell Biol.* **159**, 1109-1119.

Fukui, Y. and Inoue, S. (1997). Amoeboid movement anchored by eupodia, new actin-rich knobby feet in *Dictyostelium*. *Cel Motil. Cytoskeleton* **36**, 339-354.

Fukui, Y., de Hostos, E., Yumura, S., Kitanishi-Yumura, T. and Inoue, S. (1999). Architectural dynamics of F-actin in eupodia suggests their role in invasive locomotion in *Dictyostelium*. *Exp. Cell Res.* **249**, 33-45.

Gingell, D. (1981). The interpretation of interference-reflection images of spread cells: significant contributions from thin peripheral cytoplasm. *J. Cell Sci.* **49**, 237-247.

Gingell, D., Todd, I. and Owens, N. (1982). Interaction between intracellular vacuoles and the cell surface analysed by finite aperture theory interference reflection microscopy. *J. Cell Sci.* **54**, 287-298.

Gingell, D. and Vince, S. (1982). Substratum wettability and charge influence the spreading of *Dictyosteilm* amoebae and the formation of ultrathin cytoplasmic lamellae. *J. Cell Sci.* **54**, 255-285.

Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A., Ginsberg, M. H. and Horwitz, A. F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* **272**, 32719-32722.

Izzard, C. S. and Lochner, L. R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique. *J. Cell Sci.* **21**, 129-159.

Izzard, C. S. and Lochner, L. R. (1980). Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J. Cell Sci.* **42**, 81-116.

Keller, H. U., Zimmermann, A. and Cottier, H. (1983). Crawling-like movements, adhesion to solid substrata and chemokinesis of neutrophil granulocytes. *J. Cell Sci.* **64**, 89-106.

Kreitmeier, M., Gerisch, G., Heizer, C. and Muller-Taubenberger, A. (1995). A talin homologue of *Dictyostelium* rapidly assembles at the leading edge of cells in response to chemoattractant. *J. Cell Biol.* **129**, 179-188.

Lawson, M. A. and Maxfield, F. R. (1995). Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* **377**, 75-79.

Lee, J. and Jacobson, K. (1997). The composition and dynamics of cell-substratum adhesions in locomoting fish keratocytes. *J. Cell Sci.* **110**, 2833-2844.

Munevar, S., Wang, Y. L. and Dembo, M. (2001). Distinct roles of frontal and rear cell-substrate adhesions in fibroblast migration. *Mol. Biol. Cell.* **12**, 3947-3954.

Murray, J., Vawter-Hugart, H., Voss, E. and Soll, D. R. (1992). Three-dimensional motility cycle in leukocytes. *Cell Motil. Cytoskeleton* **22**, 211-223.

Niewohner, J., Weber, I., Maniak, M., Muller-Taubenberger, A. and Gerisch, G. (1997). Talin-null cells of *Dictyostelium* are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. *J. Cell Biol.* **138**, 349-361.

Okazaki, K. and Yumura, S. (1995). Differential association of three actin-bundling proteins with microfilaments in *Dictyostelium* amoebae. *Eur. J. Cell Biol.* **66**, 75-81.

Oliver, T., Dembo, M. and Jacobson, K. (1999). Separation of propulsive and adhesive traction stresses in locomoting keratocytes. *J. Cell Biol.* **145**, 589-604.

Palecek, S. P., Huttenlocher, A., Horwitz, A. F. and Lauffenburger, D. A. (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J. Cell Sci.* **111**, 929-940.

Palecek, S. P., Schmidt, C. E., Lauffenburger, D. A. and Horwitz, A. F. (1996). Integrin dynamics on the tail region of migrating fibroblasts. *J. Cell Sci.* **109**, 941-952.

Pelham, R. J., Jr and Wang, Y. L. (1999). High resolution detection of mechanical forces exerted by locomoting fibroblasts on the substratum. *Mol. Biol. Cell* **10**, 935-945.

Pierini, L. M., Lawson, M. A., Eddy, R. J., Hendey, B. and Maxfield, F. R. (2000). Oriented endocytic recycling of alpha5beta1 in motile neutrophils. *Blood* **95**, 2471-2480.

Regen, C. M. and Horwitz, A. F. (1992). Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J. Cell Biol.* **119**, 1347-1359.

Riveline, D., Zamir, E., Balaban, N. Q., Schwarz, U. S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. and Bershadsky, A. D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J. Cell Biol.* **153**, 1175-1186.

Ruppel, K. M., Uyeda, T. Q. and Spudich, J. A. (1994). Role of highly conserved lysine 130 of myosin motor domain. In vivo and in vitro characterization of site specifically mutated myosin. *J. Biol. Chem.* **269**, 18773-18780.

Schmidt, C. E., Horwitz, A. F., Lauffenburger, D. A., Sheetz, M. P. (1993). Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. *J. Cell Biol.* **123**, 977-991.

Seveau, S., Keller, H., Maxfield, F. R., Piller, F. and Halbwachs-Mecarelli, L. (2000). Neutrophil polarity and locomotion are associated with surface redistribution of leukosialin (CD43), an antiadhesive membrane molecule. *Blood* **95**, 2462-2470.

Shiraha, H., Glading, A., Gupta, K. and Wells, A. (1999). IP-10 inhibits epidermal growth factor-induced motility by decreasing epidermal growth factor receptor-mediated calpain activity. *J. Cell Biol.* **146**, 243-254.

Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E. and Gundersen, G. G. (1999). Focal adhesion motility revealed in stationary fibroblasts. *Science* **286**, 1172-1174.

Takubo, T. and Tatsumi, N. (1999). Microscopic co-distributions of myosin, actin, alpha-actinin and vinculin in human neutrophils during movement. *Haematologia (Budap.)* **29**, 285-294.

Todd, I., Mellor, J. S. and Gingell, D. (1988). Mapping cell-glass contacts of *Dictyosteium* amoebae by total internal reflection aqueous fluorescence overcomes a basic ambiguity of interference reflection microscopy. *J. Cell Sci.* **89**, 107-114.

Tuxworth, R. I., Weber, I., Wessels, D., Addicks, G. C., Soll, D. R., Gerisch, G. and Titus, M. A. (2001). A role for myosin VII in dynamic cell adhesion. *Curr. Biol.* **11**, 318-329.

Uchida, K. and Yumura, S. (1999). Novel cellular tracks of migrating *Dictyostelium* cells. *Europ. J. Cell Biol.* **78**, 757-766.

Uchida, K. S. K., Kitanishi-Yumura, T. and Yumura, S. (2003). Myosin II contributes to the posterior contraction and the anterior extension during the retraction phase in migrating *Dictyostelium* cells. *J. Cell Sci.* **116**, 51-60.

Wessels, D., Soll, D. R., Knecht, D., Loomis, W. F., De Lozanne, A. and Spudich, J. (1988). Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. *Dev. Biol.* **128**, 164-177.

Wessels, D., Vawter-Hugart, H., Murray, J. and Soll, D. R. (1994). Three-dimensional dynamics of pseudopod formation and the regulation of turning during the motility cycle of *Dictyostelim*. *Cell Motil. Cytoskeleton* **27**, 1-12.

Weber, I., Wallraff, E., Albrecht, R. and Gerisch, G. (1995). Motility and substratum adhesion of *Dictyostelium* wild-type and cytoskeletal mutant cells: a study by RICM/bright-field double-view image analysis. *J. Cell Sci.* **108**, 1519-1530.

Yumura, S. (1993). Reorganization of actin and myosin II in *Dictyostelium* amoeba during stimulation by cAMP. *Cell Struct. Funct.* **18**, 379-388.

Yumura, S. and Fukui, Y. (1998). Spatiotemporal dynamics of actin concentration during cytokinesis and locomotion in *Dictyostelium*. *J. Cell Sci.* **111**, 2097-2108.

Yumura, S., Furuya, K. and Takeuchi, I. (1996). Intracellular free calcium responses during chemotaxis of *Dictyostelium* cells. *J. Cell Sci.* **109**, 2673-2678.

Yumura, S. and Kitanishi-Yumura, T. (1990). Fluorescence-mediated visualization of actin and myosin fiaments in the contractile membrane-cytoskeleton complex of *Dictyostelium discoideum*. *Cell Struct. Funct.* **15**, 355-364.

Yumura, S. and Kitanishi-Yumura, T. (1992). Release of myosin II from the membrane-cytoskeleton of Dictyostelium discoideum mediated by heavy-chain phosphorylation at the foci within the cortical actin network. *J. Cell Biol.* **117**, 1231-1239.

Yumura, S., Matsuzaki, R. and Kitanishi-Yumura, T. (1995). Introduction of macromolecules into living *Dictyostelium* cells by electroporation. *Cell Struct. Funct*. **20**, 185-190

Yumura, S., Mori, H. and Fukui, Y. (1984). Localization of actin and myosin for the study of ameboid movement in *Dictyostelium* using improved immunofluorescence. *J. Cell Biol.* **99**, 894-899.

Yumura, S. and Uyeda, T. Q. P. (1997). Myosin II can be localized to the cleavage furrow and to the posterior region of *Dictyostelium* amoebae without control by phosphorylation of myosin heavy and light chains. *Cell Motil. Cytoskeleton* **36**, 313-322.

Zamir, E. and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* **114**, 3583-3590.