Moving and Stationary Actin Filaments Are Involved in Spreading of Postmitotic PtK2 Cells

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Abstract. We have investigated spreading of postmitotic PtK2 cells and the behavior of actin filaments in this system by time-lapse microscopy and photoactivation of fluorescence. During mitosis PtK2 cells round up and at cytokinesis the daughter cells spread back to regain their interphase morphology. Normal spreading edges are quite homogenous and are not comprised of two distinct areas (lamellae and lamellipodia) as found in moving edges of interphase motile cells. Spreading edges are connected to a network of long, thin, actin-rich fibers called retraction fibers. A role for retraction fibers in spreading was tested by mechanical disruption of fibers ahead of a spreading edge. Spreading is inhibited over the region of disruption, but not over neighboring intact fibers.

Using photoactivation of fluorescence to mark actin filaments, we have determined that the majority of actin filaments move forward in spreading edges at the same rate as the edge. As far as we are aware, this is the first time that forward movement of a cell edge has been correlated with forward movement of actin filaments. In contrast, actin filaments in retraction fibers remain stationary with respect to the substrate. Thus there are at least two dynamic populations of actin polymer in spreading postmitotic cells. This is supported by the observation that actin filaments in some spreading edges not only move forward, but also separate into two fractions or broaden with time. A small fraction of postmitotic cells have a spreading edge with a distinct lamellipodium. In these edges, marked actin polymer fluxes backward with respect to substrate.

We suggest that forward movement of actin filaments may participate in generating force for spreading in postmitotic cells and perhaps more generally for cell locomotion.

ACTIN filaments are thought to play a key role in cell motility. The behavior of actin filaments and actin-containing structures has been studied in a number of motile cells. In these cells actin filaments and actin-containing structures flux backward with respect to substrate (Abercrombie, 1970; Fisher et al., 1988; Forscher and Smith, 1988; Heath, 1983; Okabe and Hirokawa, 1989, 1991; Svitkina et al., 1986; Symons and Mitchison, 1991; Theriot and Mitchison, 1992; Wang, 1985), although see Theriot and Mitchison (1991). It has been thought that the force that generates this retrograde flux could be harnessed to drive protrusion. However this has not been demonstrated. We have studied actin filament dynamics in a different motile system, the spreading edges of postmitotic cells. We investigated this system for several reasons. First, the mechanism of postmitotic cell spreading has previously been little studied, and an investigation of its actin filament dynamics will increase our knowledge of actin filament behavior during different types of cell motility. Second, spread-

ing postmitotic cells share some of the morphological features found in other motile cells. Therefore an investigation of postmitotic spreading may illuminate general motile mechanisms. Finally, PtK2 cells are amenable to microinjection and microscopic observation (Mitchison, 1989, 1992; Theriot and Mitchison, 1991) and our caged-fluorescent actin probe works well in these cells (Theriot and Mitchison, 1991).

Mammalian cells in tissue culture typically round up early in mitosis (Mitchison, 1992; Sanger et al., 1984; Wetzel et al., 1978). Later, these cells must spread in order to form two daughter cells with interphase morphology. Postmitotic spreading is particularly striking in PtK2 cells. The cell bodies of dividing PtK2 cells are connected to a network of long, thin, actin-rich fibers called retraction fibers which are attached to the substrate and generally perpendicular to the bulk cell margin (Mitchison, 1992). Retraction fibers are exposed as cells round up early in mitosis (Mitchison, 1992; Sanger, 1984; Wetzel, 1978) and remain an integral part of daughters throughout spreading. During spreading, edges of postmitotic cells move over retraction fibers, the cell body gets flatter and larger, and the distance between the ends of retraction fibers and the spreading edge gets shorter.

In this paper, we first describe the morphological details

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of postmitotic PtK2 cell spreading. We then analyze actin filament dynamics in spreading edges and retraction fibers in live postmitotic cells by photoactivation of fluorescence. These dynamics provide insight into the mechanism of postmitotic cell spreading and may have implications for how actin is generally involved in driving cell locomotion.

Materials and Methods

Cell Culture, Retraction Fiber Disruption, and Microinjection

Potoroo tridactylis kidney (PtK2)[1] cells, an epithelial cell line, were obtained from the American Type Culture Collection (Rockville, MD) and grown in DME-H16 medium with 10% FCS, streptomycin, and penicillin at 37°C in 5% CO_2. 1–3 d before an experiment, they were plated onto 25-mm round glass coverslips and transferred on the day of observation to an aluminum chamber. Cells were observed at 30–32°C in bicarbonate- and phenol red-free DME, with 10% bovine calf serum, 20 mM Hepes, pH 7.4, streptomycin, and penicillin. This was covered with a thin layer of silicone oil to prevent evaporation. Microsurgical disruption/removal of retraction fibers was performed by scratching them with a glass microneedle.

Caged resorufin-actin (CR-actin) was prepared and microinjected as described (Theriot and Mitchison, 1991). Prophase or prometaphase mitotic cells were usually microinjected. These cells were not photoactivated until after a cleavage furrow was visible, the earliest time that spreading is initiated. This also ensured that CR-actin had sufficient time to incorporate into filaments before marking.

Microscopy and Data Collection

Time-lapse video recording of phase contrast images of live cells was done on a Zeiss inverted microscope (IM35) fitted with a charge-coupled device video-rate camera (Hamamatsu) with either a 40× Neofluar 0.75 NA objective and a 1× eyepiece or with a 100×, 1.4 NA oil immersion objective, and an 8× eyepiece. Paired phase and epifluorescence images of live cells for photoactivation experiments were collected less than 4 s apart using a 100×, 1.4 NA oil immersion objective and an intensified silicon-intensified target tube camera. All live images were recorded on an optical disc recorder (Panasonic, Secaucus, NJ) controlled by Maxvision (Datacube, Peabody, MA). Photoactivation was performed using apparatus as described (Mitchison, 1989) for 600–900 ms depending on the resultant intensity of the epifluorescent signal. Resorufin was imaged with a 577 df20

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1. *Abbreviations used in this paper*: CR-actin, caged resorufin-actin; PtK2, potoroo tridactylis kidney; TX, Triton X-100.

nm bandpass excitation filter and a 622df65 nm bandpass emission filter. The mercury arc light source was attenuated with either a 12 or 87% transmission filter (usually with 12%). In both cases photobleaching of resorufin was not significant. A red filter was placed in front of the transmitted light path. Image analysis and enhancement of intensified silicon-intensified target images for photography were performed using Image 1 version 4 software (Universal Imaging Corp., West Chester, PA). Rate measurements, turnover and photobleaching determinations, and fluorescent intensity profiles were obtained from unenhanced images. Images stored on optical disc were photographed from a flat screen monitor with TMAX or Plus-X film (Kodak).

### Fluorescent Staining of Actin Filaments

Cells on glass coverslips were fixed in cytoskeleton buffer (Small, 1981; Symons and Mitchison, 1991) with 4% formaldehyde and 0.32 M sucrose for 20 min. Cells were then washed with cytoskeleton buffer and permeabilized with 0.5% Triton X-100 (TX) in cytoskeleton buffer for 10 min. After washing in TBS-TX (0.15 M NaCl, 0.02 M Tris-Cl, 0.1% TX, pH 7.4) actin filaments were stained with 1 μg/ml rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) in TBS-TX for 15–30 min. Cells were then washed in TBS-TX, rinsed in TBS, dunked in distilled water, and mounted in antifade (FITC-guard, Testog Inc., Chicago, IL). Cells were imaged on a Zeiss photomicroscope with hypersensitized Technical Pan film (Kodak).

Results

General Properties of Spreading Postmitotic Cells

Postmitotic PtK2 cells start to spread in telophase, when at least the beginnings of a cleavage furrow are visible. Spreading continues through cytokinesis and takes ∼1–2.5 h (at 30–32°C). Fig. 1 illustrates spreading shortly after cytokinesis (early stage, Fig. 1 a) through to formation of two daughter cells with interphase morphology (late stage, Fig. 1 c). These spreading stages occur in all spreading postmitotic cells. However, the overall appearance of individual spreading cells is highly heterogenous, with individual edges ranging in appearance from branched to uniform morphology (see below).

During the early stage of spreading (Fig. 1 a), daughters are characteristically round. Dividing cells are connected to a network of long, thin fibers (retraction fibers) which are also attached to the substrate (e.g., arrow in Fig. 1 a). During the intermediate stage of spreading (Fig. 1 b) daughters
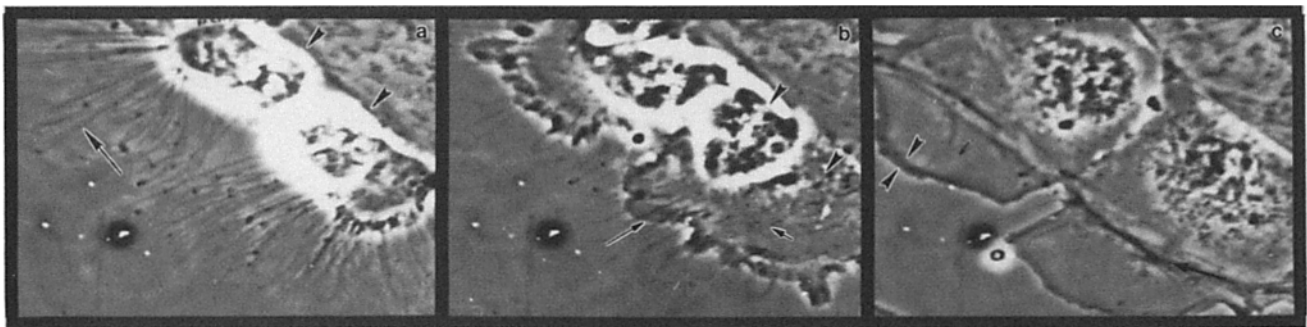
Figure 1. General properties of postmitotic cells. (*a–c*) Low power high contrast phase images of a spreading, postmitotic cell. In each panel daughters are spreading from top to bottom. Portion of an interphase cell lies above the postmitotic cell (touching the two daughters). (*a*) Early stage of spreading. Daughters (*arrowheads*) are characteristically round. Retraction fibers are denoted with the arrow. (*b*) Intermediate stage of spreading. Daughters begin to flatten out more. The spreading edge of the lower daughter lies between the long and short arrow. Two arrowheads point to the main body and organelle-rich area of this daughter. The latter extends to the short arrow. (*c*) Late stage of spreading. Daughters take on an interphase appearance. A lamella (*short arrow*), lamellipodium (*between the arrowheads*), and circumferential ring (*long arrow*) form. Bar, 10 μm.

begin to flatten out more. The spreading edge is quite homogenous (between the *arrows*, Fig. 1 *b*) and lacks the differentiation into two distinct structures (lamellae and lamellipodia) that is typical of fibroblast leading edges (Heath and Holifield, 1991). Towards the end of spreading, daughters take on an interphase appearance (Fig. 1 *c*). This starts to occur when a spreading daughter is close to, or reaches the distal ends of retraction fibers. At this late stage of spreading, a lamella (*short arrow*, Fig. 1 *c*) and lamellipodium (between the *arrowheads*, Fig. 1 *c*) become visibly differentiated. Also apparent in Fig. 1 *c* is a circumferential ring which also forms during the late stage of spreading (*long arrow*). Circumferential rings are a common feature of epithelial cells. Formation of an interphase cell has been arbitrarily set as the end of spreading, although daughters can still be ~0.3–0.5-fold smaller than older interphase cells. The final size of newly divided cells depends on cell density.
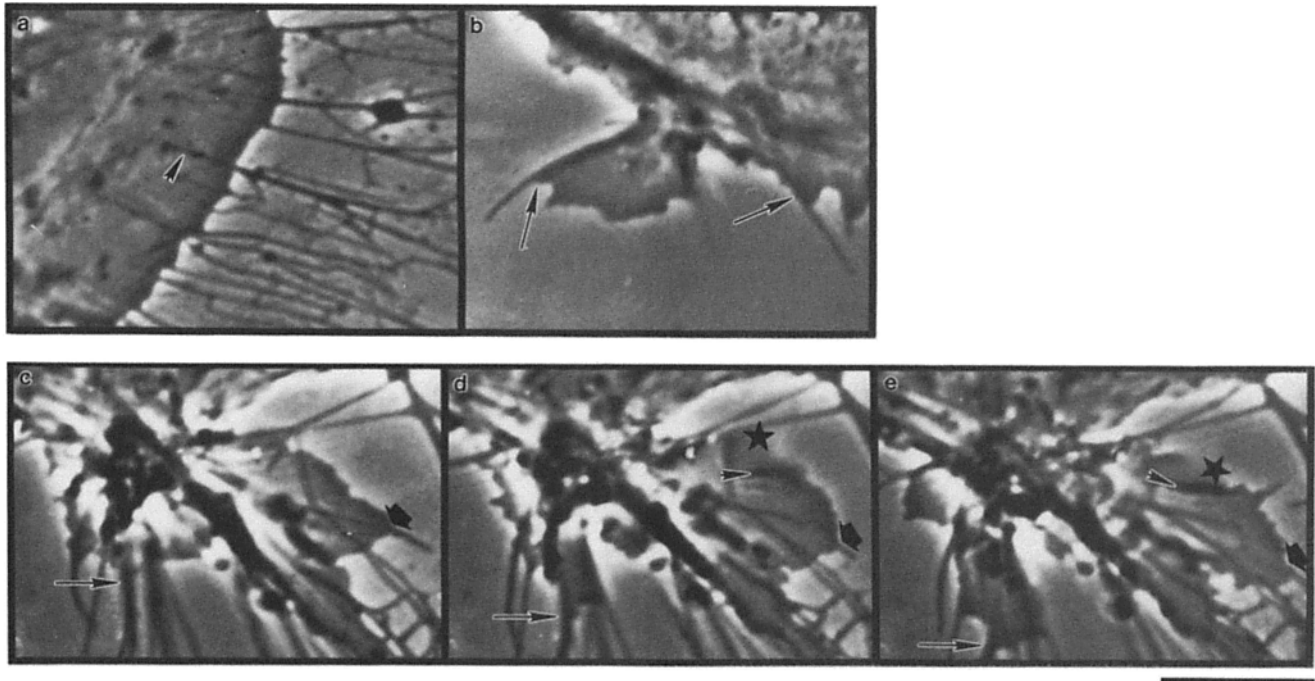
Postmitotic Cells Spread over Retraction Fibers and Extend Filopodia

When retraction fibers emanate from a given region of a spreading edge, the edge spreads smoothly over them (Fig. 2 *a*). Note that retraction fibers do not terminate at the spreading edge, but persist as phase-dense fibers within the cell (Fig. 2 *a, arrowhead*). Smooth spreading edges do not usually extend long, thin, actin structures called filopodia. However, when retraction fibers do not emanate from a given region of a spreading edge (they may lie parallel to that

edge), or are sparse, or absent, the edge tends to generate filopodia (Fig. 2 *b*) and can ruffle (Fig. 2, *d–e*) as it spreads. Filopodia either protrude, and then attach to substrate, or protrude directly along the substrate. They are distinguished from retraction fibers since they are generated by protrusion rather than retraction, and are shorter and appear to have a more uniform density by phase contrast microscopy. Spreading smoothly over retraction fibers and formation of filopodia can occur simultaneously in different regions of a spreading cell, depending on the position and density of retraction fibers as shown by time-lapse images in Fig. 2, *c–e*. The *thin arrow* in Fig. 2 *c* points to a cluster of seven or eight retraction fibers over which an edge spreads smoothly (*thin arrows*, Fig. 2, *d–e*). The *thick arrows* in Fig. 2, *c–e* point to another spreading edge from which retraction fibers mostly do not emanate (only two present on the left side). This edge generates a filopodium (*thick arrows*, Fig. 2, *c–e*) and ruffles (*asterisk* at a diffuse area and *arrowhead* at a folding over piece of membrane in Fig. 2, *d* and *e*). Postmitotic cells spread over retraction fibers up to fourfold faster than edges which spread without emanating retraction fibers. Thus spreading at clusters of retraction fibers is more likely to result in a branched morphology (Fig. 2, *c–e*). In contrast to Fig. 2 *a*, retraction fibers are high in density and evenly spaced which promotes a uniform spreading edge.

Actin Organization in Postmitotic Cells

Fig. 3 shows that daughters throughout all stages of spread-

Figure 2. Postmitotic cells spread over retraction fibers and extend filopodia. (*a–e*) High power high contrast phase images of spreading, postmitotic cells. The daughters are spreading from left to right in *a* and from top to bottom in *b–e*. (*a*) An edge spreading smoothly over retraction fibers with no observable formation of filopodia. Note the regular spacing between retraction fibers which promotes a uniform spreading edge. Also note persistence of retraction fibers within the spreading edge (e.g., *arrowhead*). (*b*) An edge spreading at sites of filopodia formation (*arrows*). Note the absence of retraction fibers. (*c–e*) An edge spreading smoothly (*thin arrows*; *d* and *e*) over retraction fibers (*thin arrow*; *c*) at an average instantaneous rate of 1.82 μm/min (*c–e* spans 132 s = 2.2 min). Note that clusters of retraction fibers generate a branched spreading edge. Another edge extends a filopodium (*thick arrows*; *c–e*), ruffles (*asterisk* at a diffuse area and *arrowhead* at a folding over piece of membrane; *d* and *e*), and spreads at an average instantaneous rate of 0.75 μm/min. Bar, 10 μm.
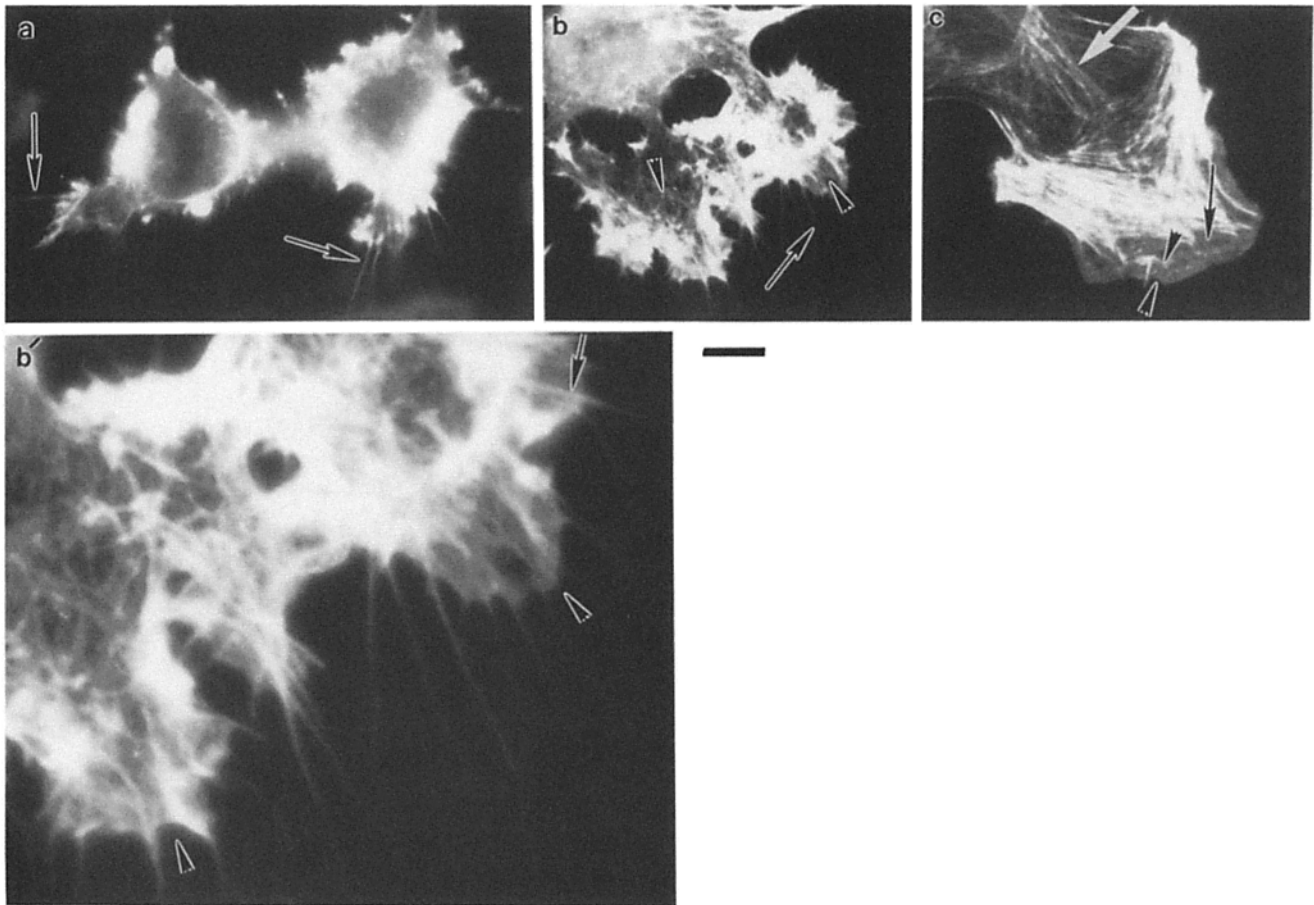
Figure 3. Actin organization in postmitotic cells. PtK2 cells were fixed, permeabilized, and stained with rodamine-phalloidin. (*a*) Early stage of spreading. Actin polymer in retraction fibers is indicated with arrows. (*b*) Intermediate stage of spreading (one daughter shown). A filamentous actin network can be identified (*arrowheads*). Actin polymer in retraction fibers is again noted (*arrow*). *b* is seen in more detail at higher magnification (*b'*). In *b'*, note actin in retraction fibers persists inside the spreading edge (e.g., *arrow*), and clusters of retraction fibers promote branched spreading (e.g, two *arrowheads*). (*c*) Late stage in spreading (portion of one daughter shown). Stress fibers (*white arrow*), a lamella (*black arrow*), and lamellipodium (between the *arrowheads*) form. Bars: (*a–c*) 10 µm; (*b'*) 3 µm.

ing contain a considerable amount of actin polymer. Early in spreading actin filaments are abundant, but their organization is unclear because daughters are very round (Fig. 3 *a*). As observed previously (Mitchison, 1992), actin polymer is found in retraction fibers (Fig. 3 *a, arrows*). By the intermediate stage of spreading, a filamentous actin network can be identified (Fig. 3 *b, arrowheads*). Fig. 3 *b* is seen in more detail at higher magnification in Fig. 3 *b'*. Note here that actin filaments are found at the very edges of spreading cells. In contrast, microtubules are not found at the very edges (not shown). Notice that actin in retraction fibers can be perpendicular to the spreading edge and remains as such inside the cell (*arrow*, Fig. 3 *b'*). Also illustrated in Fig. 3 *b'* are clusters of retraction fibers promoting branched spreading (e.g., *arrowheads*, Fig. 3 *b'* and as previously shown in Fig. 2, *c–e*). Stress fibers disappear at the onset of mitosis and do not reform until the daughter regains an interphase morphology during the later stages of spreading (Fig. 3 *c, white arrow*). An interphase lamella (*black arrow*) and a lamellipodium (between the *arrowheads*) also form at this stage (Fig. 3 *c*).

Mechanical Disruption of Retraction Fibers Inhibits Spreading of Individual Edges

To investigate a possible role for retraction fibers in spreading, fibers were disrupted using a glass microneedle. In 11 manipulated daughter cells, spreading of individual edges over disrupted groups of retraction fibers was inhibited (14/14 edges, 8 of which also exhibited slight retraction), while spreading over neighboring intact fibers was not affected (12/14 cases). The 2/14 edges which stopped at the disrupted site but did not continue spreading over intact neighboring fibers were unable to do so because other edges were spreading over these fibers. This selective inhibition of spreading changed the overall direction of spread of the individual edges (Fig. 4).

Inhibition of spreading occurred either immediately or after a short time of continued spreading, depending on the distance between the spreading edge and the region of disruption. That spreading continues to the site of disruption (as occurs from Fig. 4, *a–b* to the *white asterisks*) indicates that retraction fibers between the spreading edge and the dis-
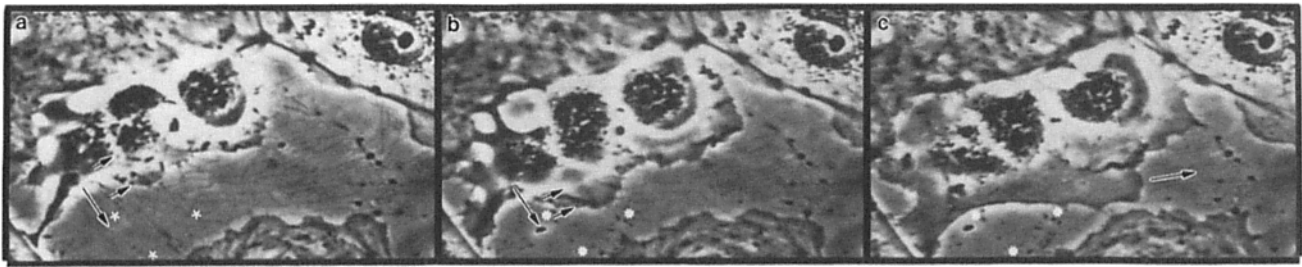
Figure 4. Mechanical disruption of retraction fibers inhibits spreading of individual edges. Low power high contrast phase images of a spreading postmitotic cell. (*a*, 385 s). The left hand daughter is spreading in the direction of the long arrow at 1.39 μm/min (0–385 s). The area between the two short arrows indicates the extent of spread that has occurred by 385 s. White asterisks outline the retraction fiber region disrupted in *b*. (*b*, 616 s). Retraction fibers were microsurgically disrupted over a maximum of 165 s (451–616 s) ahead of the spreading edge (*outlined with white asterisks*). Spreading continues to the site of disruption at 1.46 μm/min (385–616 s = 231 s) in the same direction as before disruption (*long arrow*). The area between the two short arrows indicates the extent of spread that has occurred over 385–616 s. (*c*, 1,089 s). Spreading is prevented over disrupted fibers (*white asterisks*) but not over neighboring intact fibers (616–1,089 s = 473 s) which changes the overall direction of spread (in the direction of the *arrow* at 2.38 μm/min). The density of retraction fibers within the white asterisks in *a* is the same as the density of fibers within a same area in the new direction of spread in *c*. Rates were measured over a range of 231–473 s (3.9–7.9 min). Rates of spreading are higher than those in Figs. 5–7 because disruption experiments were performed at a higher temperature. Bar, 10 μm.

rupted site are not affected by the microsurgery. It is possible that damage to any extracellular matrix, as a result of the disruption procedure, could affect spreading. However, scratching the coverslip with glass microneedles, without causing damage to retraction fibers, did not prevent spreading nor change the morphology of spreading (not shown).

Once edges had stopped spreading at disrupted retraction fiber sites their behavior was observed for up to 30 min. In 7/14 cases, inhibition of spreading persisted. The other seven edges resumed spreading over or towards the disrupted site after a lag of an average of ~10 min. These spreading edges which now lacked retraction fibers and ruffled and extended filopodia. If all retraction fibers of a spreading daughter are removed, the spreading edge extends filopodia and ruffles. There is no lag in spreading, but the rate of advance is 1.5–2-fold slower than before removal of all retraction fibers.

Actin Filaments Move Forward in Spreading Edges

An unperturbed actin filament system is required for spreading of postmitotic cells, because cytochalasin D treatment reversibly inhibits spreading (not shown). To investigate the dynamics of actin polymer in spreading edges, PtK2 cells were microinjected with actin subunits covalently modified with CR-actin. Cells were microinjected early in mitosis at prophase or prometaphase, or during spreading, allowing at least 25 min for CR-actin to incorporate into filaments before photoactivation of fluorescence. After photoactivation, the behavior of actin filaments was compared to that of the spreading edge by collecting paired epifluorescence and phase images with an intensified silicon-intensified target camera. In 54/54 cells examined during the early or intermediate stage of spreading, some or all of the observable marked actin filaments in spreading edges moved forward relative to substrate at the same rate as the edge (Fig. 5). Line intensity profiles readily demonstrate movement of fluorescently marked filaments with time (Fig. 5 *g*). Actin behavior is tightly coupled to edge behavior as shown in the graph in Fig. 5 *h*. The high correlation between actin polymer and edge behavior was not dependent on distance from the spreading edge; the same result was obtained up to 13 μm from the edge (distances greater than 13 μm were not studied).

Actin Filaments are Stationary in Retraction Fibers during Spreading

Marks were made on actin filaments in retraction fibers that were perpendicular to a spreading edge by photoactivation of fluorescence. Actin filaments in these fibers were stationary with respect to substrate (43/50 cases=86%). Actin filaments in the other 7/50 cases moved forward (14%), but in five of these, a fraction of the mark remained stationary resulting in a separation or broadening of actin filaments. Thus in 48/50 retraction fibers (96%) all or some of the actin filaments remained stationary with respect to substrate. In 10 cases we were able to continue monitoring the behavior of the stationary retraction fiber actin filaments after the edge had spread over the marked fiber region. As shown in Fig. 6, actin filaments remained stationary with respect to substrate within the spreading edge (9/10 cases). In the other case, the actin polymer moved forward at the same rate as the spreading edge.

Marked Actin Filaments Separate in Spreading Edges

Actin in retraction fibers penetrates the spreading edge (Figs. 2 *a* and 3 *b'*) and is stationary with respect to substrate during spreading, before and after the edge has spread over the marked fiber region (Fig. 6). Thus we expected that at least some actin polymer would remain stationary when marked in spreading edges. This does occur. Marked zones on actin filaments separated (majority) or broadened (minority) in 52% (28/54 observations, same data set as Fig. 5) of postmitotic cell edges that had been microinjected with CR-actin and marked during spreading. This occurred over the entire marked zone or in one part of the zone and comprised roughly 25% or less of the total actin marked. A smearing of signal between separating populations of filaments was also observed about half the time. The spreading daughter in Fig. 7 is moving forward from left to right. Some actin
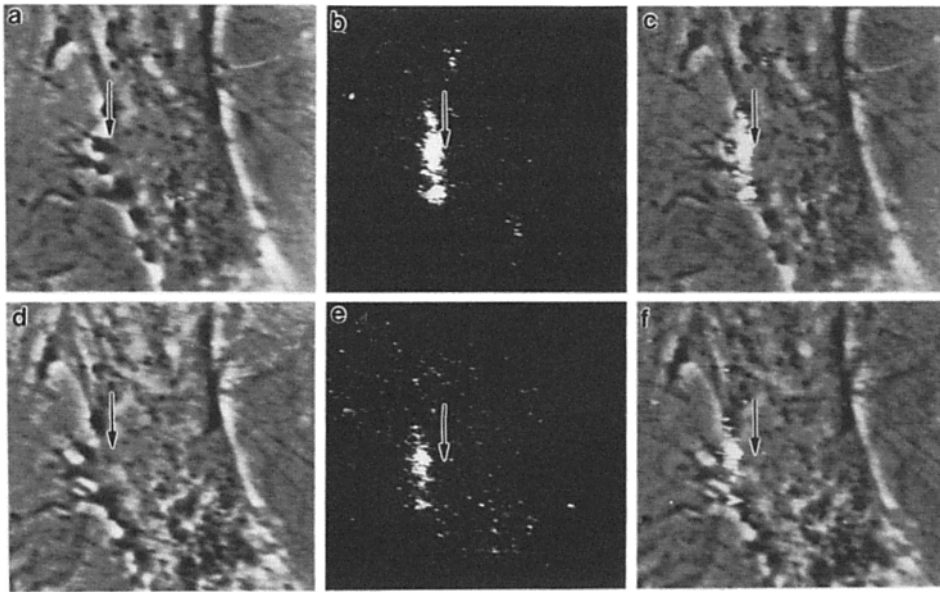
Figure 5. Actin filaments move forward in spreading edges. CR-actin filaments were marked by photoactivation and observed in a postmitotic spreading edge. (*a–f*) This spreading daughter is moving forward at 0.13 μm/min from right (*a* and *c*) to left (*d* and *f*) (portion of one daughter shown). Actin filaments also move forward, relative to substrate, at 0.12 μm/min (*b* and *c* = 374 s after photoactivation, *e* and *f* = 1,056 s after photoactivation, the spreading edge was moving about threefold slower up to 374 s after photoactivation). Arrows in *a–f* indicate fixed points in *a–c*, respectively. Composite phase and epifluorescence images are illustrated in *c* and *f.* Portion of an interphase cell is present to the left of the spreading daughter. (*g*) Line intensity profiles of marked actin filaments in *b* and *e* (*dashed line* = 374 s after photoactivation; *solid line* = 1,056 s after photoactivation). (*h*) Quantitation of actin polymer motility vs forward edge movement. Since actin filaments can separate during spreading (Fig. 7) forward, moving actin polymer was measured in each case. This actin behavior is tightly coupled to edge behavior of spreading daughters. Slope of the graph is y = x with regression coefficient of 0.91. The average rate of forward movement (individual rates were measured over a range of up to 23 min) is 0.14 μm/min for actin polymer (range 0.01–0.45 μm/min, SD 0.10, n = 54) and 0.14 μm/min for the spreading edge (range 0.01–0.40 μm/min, SD 0.09, n = 54). Instantaneous rates of forward movement (measured over 1–2 min) can reach up to 2.1 μm/min (not plotted). Average actin filament turnover = 432 s (measured as a function of mean fluorescence intensity), n = 10; SD = 206. Bars: (*a–f*) 10 μm; (*g*) 1 μm.

filaments also move forward, relative to substrate, at the same rate (Fig. 7, *b, c, e,* and *f*). However in the lower part of the mark, a proportion of actin polymer appears to remain stationary resulting in a splitting of the mark into two components (compare Fig. 7, *b–e* and *c–f*). Line intensity profiles (taken through the lower part of the mark represented with a *broken black line* in 7 *f*) demonstrate the apparent separation of fluorescently marked filaments into two peaks with time (Fig. 7 *g*). Separation and broadening of marked actin filaments either occurred by some actin moving forward and other remaining stationary (over half of the observations), or some moving forward and the other backward (under half of the observations). These observations were not dependent on distance from the edge (observed up to 11 μm away).

Marked Actin Filaments Can Also Flux Centripetally in Spreading Edges

The most documented behavior of actin filaments and actin-containing structures in peripheral regions of motile cells is centripetal movement relative to substrate (Abercrombie, 1970; Fisher et al., 1988; Forscher and Smith, 1988; Heath, 1983; Okabe and Hirokawa, 1989, 1991; Svitkina et al., 1986; Symons and Mitchison, 1991; Theriot and Mitchison, 1992; Wang, 1985). This has been extensively studied in lamellipodia and is in contrast to the forward movement of actin filaments observed here in spreading edges of postmitotic cells. Spreading postmitotic cells do not usually comprise a lamellipodium as found in interphase motile cells. If a lamellipodium does form before the late stage of spreading,
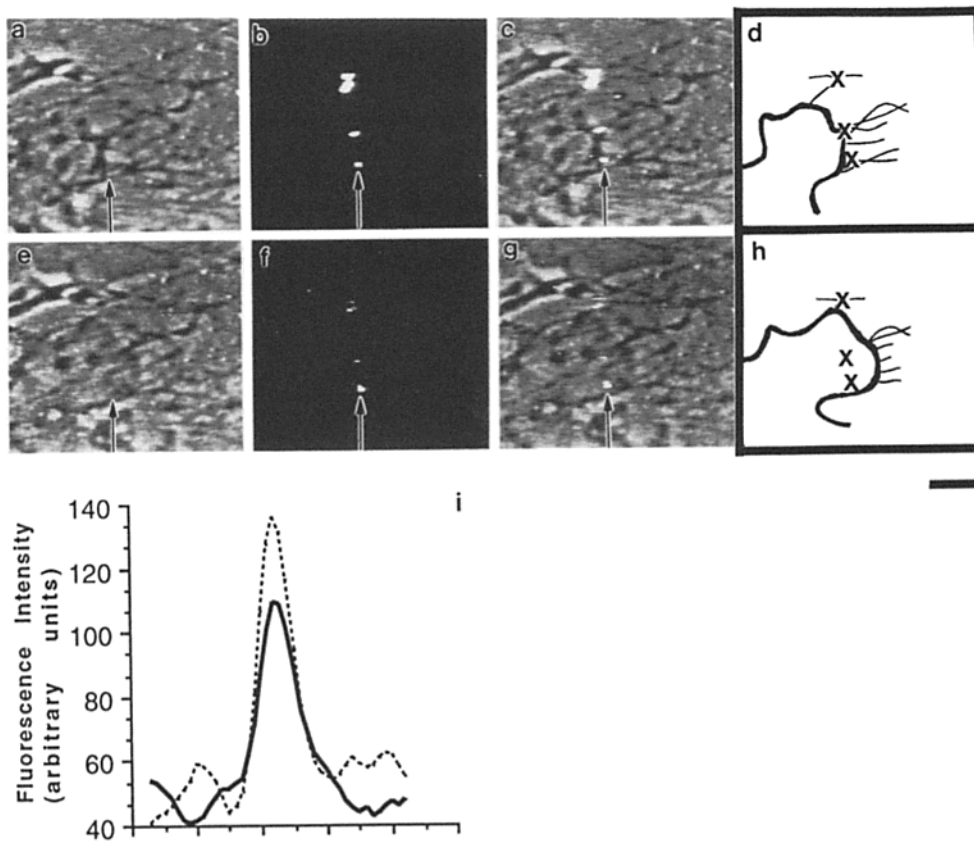
Figure 6. Actin filaments remain stationary in retraction fibers during spreading. (*a–h*) This spreading edge (outlined with a *thick black line* in *d* and *h*) is moving forward at 0.68 μm/min from left (*a–c*) to right (*e–g*) (portion of one daughter shown). Portions of some retraction fibers are denoted with *thin black lines* in *d* and *h*. Actin filaments marked in these retraction fibers remain stationary relative to substrate (measured over 14.7 min) (*b* and *c* = 14 s after photoactivation, *f* and *g* = 893 s after photoactivation). Marks were made ahead (*upper most mark* in *b* and *upper cross* in *d* and *h*) and just ahead (*lower two marks* in *b* and *lower crosses* in *d* and *h*) of the spreading edge. Arrows in *a–c* and *e–g* and crosses in *d* and *h* indicate fixed points in *a–d*, respectively. Composite phase and epifluorescence images are shown in *c* and *g*. (*i*) Line intensity profiles of marked actin filaments of the lower most mark in *b* and *f* (*dashed line* = 14 s after photoactivation, *solid line* = 893 s after photoactivation). Average actin filament turnover = 508 s (measured as a function of mean fluorescence intensity), *n* = 8; SD = 275 s. Bar, 10 μm.

Discussion

### Organization of Spreading Edges and Role of Retraction Fibers

During spreading, daughters change from a round to a flat morphology with time, as described by three major stages (Fig. 1). Spreading edges of these daughters contain a dense actin meshwork (Fig. 3, *b* and *c*) as found in edges of other motile cells (Hoglund et al., 1980; Letourneau, 1983; Lewis and Bridgman, 1992; Rinnerthaler et al., 1991; Small, 1981). Spreading postmitotic cells exhibit a number of mor-

it is usually small and short-lived. However, in a few cases, large, stable lamellipodia were observed in postmitotic cells that exhibited protrusive activity. Occurrence of such lamellipodia (which were present before the daughter became interphase overall) coincided with rare cases of postmitotic cells having vastly reduced numbers of retraction fibers. When actin filaments were marked in this type of edge, they moved centripetally relative to substrate (Fig. 8). Two other such cases (in one other cell) gave similar rates and are also similar to rates of lamellipodial protrusion and centripetal flow of actin filaments in interphase PtK2 lamellipodia (not shown). Thus PtK2 interphase-like lamellipodia that occasionally form in PtK2 cells before the late stage of spreading show actin dynamics which are different from those in edges of typically spreading postmitotic cells.

phological features including retraction fibers (Figs. 1–3). Actin in these retraction fibers penetrate into spreading edges as filaments perpendicular to the cell margin (Figs. 2 *a* and 3 *b'*). The organization of retraction fiber actin (Mitchison, 1992) may be similar to that of microspikes and filopodia (Hoglund et al., 1980; Letourneau, 1983; Small, 1981).

Retraction fibers emanate from mitotic (Mitchison, 1992; Sanger et al., 1984) and early and intermediate postmitotic PtK2 cells (Figs. 1–3). They have also been observed in mitotic and postmitotic CHO cells (Wetzel et al., 1978) and primary fibroblasts (our unpublished observations) growing in tissue culture on 2D-substrates. We have also noticed that they are present in mitotic and postmitotic primary fibroblasts growing in a 3D-collagen gel and so are probably a common feature of dividing cells that normally grow attached to a substrate, although we have not looked at cells in situ. In postmitotic PtK2 cells, retraction fibers probably have a role in spreading. This is because disruption of retraction fibers inhibits spreading at the disrupted site and promotes continued spreading in a new direction over neighboring intact fibers, rather than continued spreading at a slower rate without stoppage over the disrupted fibers, as occurs if the entire population of retraction fibers is removed (Fig. 4 and accompanying text).

Forward, Stationary, and Backward Behavior of Marked Actin Filaments in Spreading Edges and Retraction Fibers

In normal spreading postmitotic cells, actin filaments move
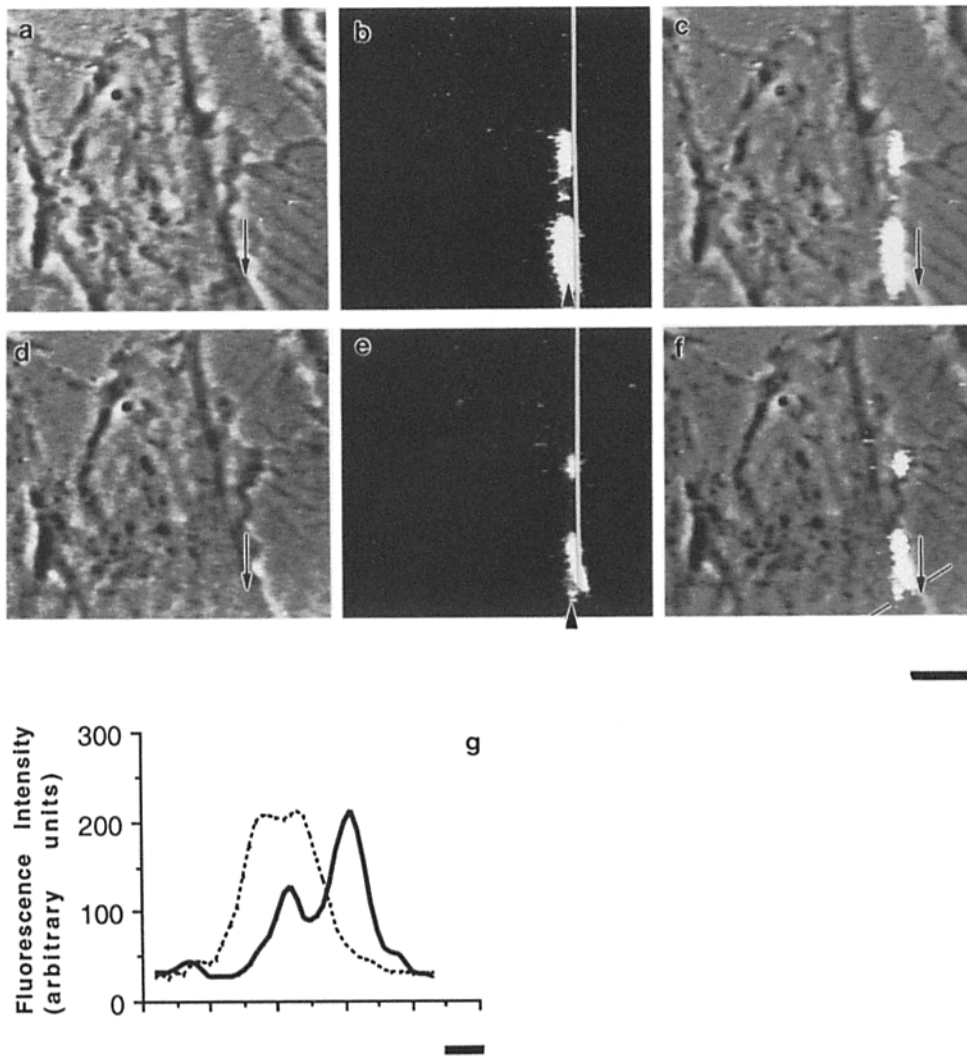
Figure 7. Marked actin filaments separate during spreading. (*a–f*) This spreading daughter is moving forward at 0.17 μm/min from left (*a* and *c*) to right (*d* and *f*) (measured over 10.6 min) (portion of one daughter shown). Some actin filaments also move forward, relative to substrate, at the same rate, as shown by filaments at the bottom of the mark moving to the right of the vertical white line in *e* (*b* and *c* = 11 s after photoactivation, *e* and *f* = 648 s after photoactivation). Other actin polymer at the bottom of the bottom mark appears to remain stationary relative to substrate (*arrowheads* in *b* and *e*) resulting in a separation of the mark. Arrows (*a, c, d,* and *f*), arrowheads (*b* and *e*), and a vertical white line (*b* and *e*) indicate fixed points in *a–c,* respectively. Composite phase and epifluorescence images are shown in *c* and *f.* (*g*) Line intensity profiles of marked actin filaments in *b* and *e* (*dashed line* = 11 s after photoactivation; *solid line* = 648 s after photoactivation). Profiles were taken approximately along the axis represented with a broken black line in *f.* Bars: (*a–f*) 10 μm; (*g*) 1 μm.

forward and remain stationary. In a small fraction of spreading edges, F-actin moves backward. This suggests that these cells contain more than one dynamic population of actin filaments.

The majority of actin filaments move forward relative to substrate in edges of early and intermediate spreading daughters (Figs. 5 and 7). As far as we are aware, this is the first time that actin filaments have been documented to be moving forward in an unperturbed motile system.

Actin filaments are stationary with respect to substrate in retraction fibers (Fig. 6). Retraction fiber actin persists within a spreading edge (Figs. 2 *a* and 3 *b'*) and remains stationary in that edge (Fig. 6). This probably explains why not all actin filaments marked in some spreading edges move forward, but some remain stationary resulting in a separation or broadening of filaments (Fig. 7). We expect separation or broadening of actin filaments to occur in all spreading edges, but in some edges, the signal from the stationary actin filaments is too low to be detected by our system.

Actin polymer moves backward with respect to substrate in early and intermediate spreading cells that bear large, interphase-like lamellipodia (Fig. 8). It is very rare for such lamellipodia to form before the late stage of spreading.

Therefore such retrograde actin filament flux is unlikely to be involved in generating force for movement in typically spreading cells. In other motile cells retrograde flux with respect to substrate occurs in lamellipodia, but its relevance for forward cell movement has not been demonstrated (Abercrombie, 1970; Fisher et al., 1988; Forscher and Smith, 1988; Okabe and Hirokawa, 1989, 1991; Svitkina et al., 1986; Symons and Mitchison, 1991; Theriot and Mitchison, 1992; Wang, 1985). Our data indicates that forward transport of actin must also be considered in models for cell locomotion.

A Simple Model for Forward Movement of Actin Filaments and Spreading

We propose one model for forward movement of actin filaments and spreading that best fits our current data. Retraction fibers are attached to the ventral substrate. We envisage that adhesion molecules immobilize retraction fiber actin polymer and at least some ventral F-actin in the spreading edge to the ventral substrate. This would account for the stationary behavior of both actin filaments in retraction fibers (Fig. 6), and of that of a proportion of actin filaments in some
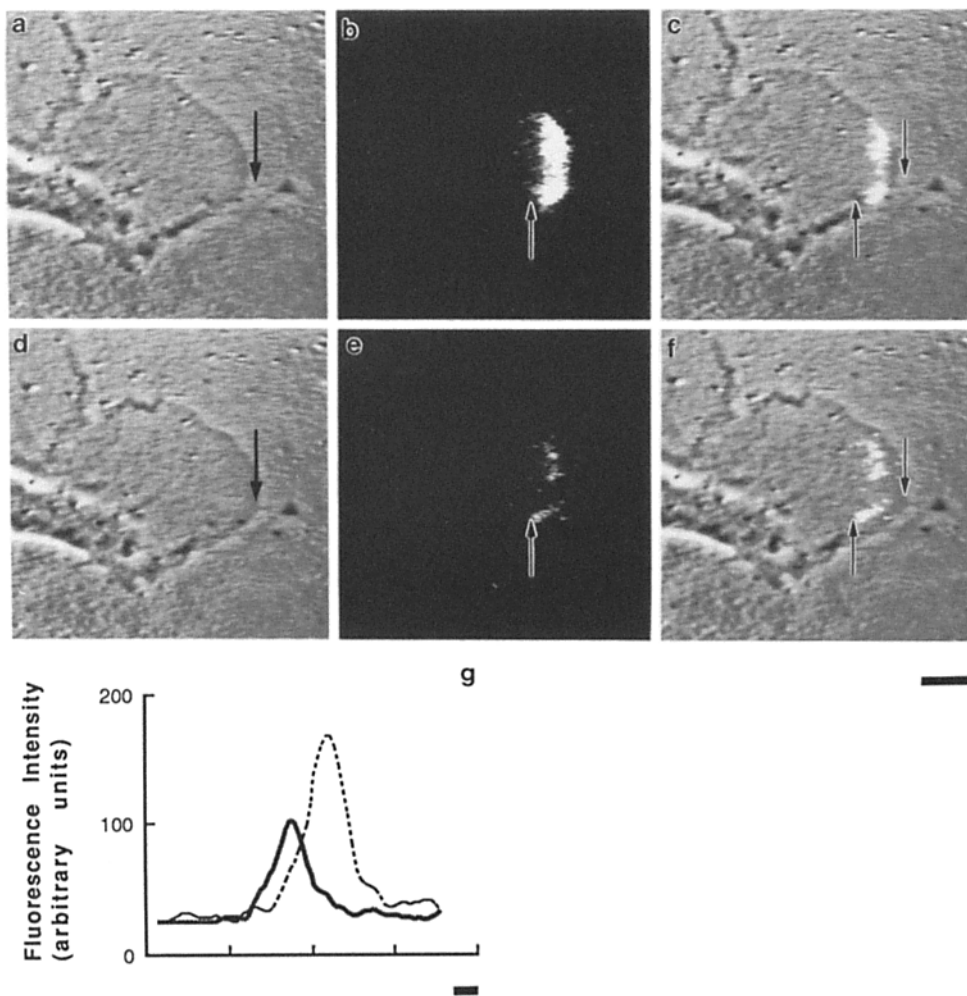
Figure 8. Actin filaments can also flux centripetally in spreading edges. (*a–f*) This large lamellipodium is moving forward at an instantaneous rate of 0.53 μm/min from left (*a* and *c*) to right (*d* and *f*). Actin filaments move backward relative to substrate at an instantaneous rate of 1.05 μm/min (*b* and *c* = 11 s after photoactivation; *e* and *f* = 90 s after photoactivation). Arrows in *a–f* mark fixed points in *a–c*, respectively. (*g*) Line intensity profiles of marked actin filaments in *b* and *e* (*dashed line* = 11 s after photoactivation; *solid line* = 90 s after photoactivation). Profiles were taken across the bottom region of the mark. Bars: (*a–f*) 10 μm; (*g*) 1 μm.

spreading edges (Fig. 7). Some actin filaments marked in edges move backward in some cases of separation or broadening of actin polymer (text accompanying Fig. 7). If these also represent ventral filaments that can stick to substrate, then sticking to substrate may not occur continuously within the edge for all ventral polymer. The rest of the spreading edge actin polymer (which moves forward at the same rate as the edge, Fig. 5) would comprise more dorsal actin filaments. We propose that these more dorsal actin filaments move forward over immobilized ventral actin polymer with resultant spreading. In this model, barbed-end directed myosins track outward over the ventral actin fibers, pulling more dorsal actin filaments forward (Fig. 9). This requires at least some of the ventral actin filaments to be orientated with their barbed ends toward the spreading edge. This polarity predominates in other motile cell systems (Lewis and Bridgman, 1992; Mooseker and Tilney, 1975; Small, 1978; Symons and Mitchison, 1991) and we are currently investigating the orientation of actin filaments in spreading postmitotic cells. The myosin would have to have two binding sites for actin as in filaments of myosin II or individual molecules of some myosin Is (Cheney and Mooseker, 1992). Preliminary data on the distribution of myosin II in spreading postmitotic cells, is consistent with this view.
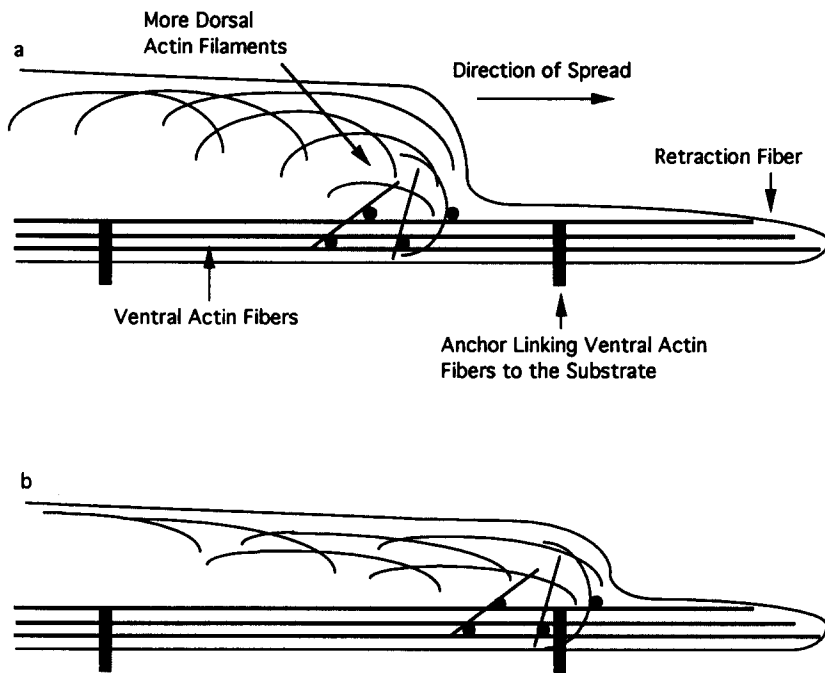
This model does not invoke a role for actin assembly in spreading, yet we know that cytochalasin D which results in the depolymerization of F-actin reversibly inhibits spreading. How can this apparent conflict be resolved? First, one action of cytochalasin is to bind to the barbed end of actin filaments. This may cause the detachment of any filaments that are normally associated with the plasma membrane and/or the substrate. Thus immobilized ventral F-actin as described in our model may detach from the substrate in the presence of cytochalasin. Traction force could then not be generated. Second, actin polymerization may be required to provide more immobilized ventral actin polymer and/or more dorsal polymer for forward movement, at least later in spreading. Early in spreading, postmitotic cells stain brightly for actin polymer and filaments have a relatively long half-life (7–8.5 min), so F-actin is already available for forward movement.

We also do not address a role for adhesion in spreading. Unfortunately, at this time we are unable to quantitatively or mechanistically address whether adhesion contributes to providing force for spreading, or whether it is responsible for maintaining the spread shape after the edge has advanced.

Relevance of Spreading for Other Motile Systems

We compare spreading edges to one other motile structure

a
More Dorsal
Actin Filaments

Direction of Spread

Retraction Fiber

Ventral Actin Fibers

Anchor Linking Ventral Actin
Fibers to the Substrate

b

Figure 9. A simple model for forward movement and stationary behavior of actin filaments and spreading. (*a*) Ventral actin filaments within the spreading edge and retraction fiber actin are continuous or overlapping with each other (*ventral actin fibers* = *horizontal thick black lines*). Anchors (*two short vertical black bars*) immobilize ventral actin fibers to the substrate. Putative myosins (*small black circles*) link more dorsal actin filaments (*curved thin black lines*) to ventral actin fibers. Ventral actin is drawn longer than dorsal actin, in keeping with observations made in growth cones (Lewis and Bridgman, 1992). (*b*) Myosins move forward along immobilized ventral actin fibers (F-actin remains stationary with respect to substrate) pulling along more dorsal actin filaments (F-actin moves forward with respect to substrate) with resultant spreading.

(growth cones) and discuss the relevance of our data for cell migration in general. Spreading of postmitotic cells seems to be similar to neuronal outgrowth in that both cell types exhibit movement away from a more fixed point. Four observations suggest that spreading postmitotic cells resemble the appearance, behavior, and actin filament organization of certain types of growth cones. First, some growth cones extend numerous filopodia and this resembles the detailed morphology of spreading cells with retraction fibers replacing the filopodia (Figs. 2, *a, c–e* and 3 *b'*). Second, in some growth cones, thin veils of cytoplasm are found between filopodia and these are also found between some retraction fibers. The occurrence of veils between retraction fibers depends on retraction fiber spacing (e.g., they are not observed in Fig. 2 *a* where retraction fibers are dense and uniformly spaced, but are observed in Fig. 2, *d–e* where retraction fibers are clustered). Third, displacement of microspikes, single veils or whole sides of growth cones induces growth cone steering (Wessells and Nuttall, 1978) and this could be similar to reorientation of spreading after removal of a subset of retraction fibers (Fig. 4). Finally, ultrastructural studies suggest that two populations of actin filaments exist in growth cones (Lewis and Bridgman, 1992) and this is consistent with there being more than one dynamic population of actin filaments in postmitotic spreading cells (Figs. 5–8).

The observation of forward movement and stationary behavior of actin polymer in postmitotic cells may help the way in which we generally think about cell migration. One function for lamellipodia, filopodia, pseudopodia, and retraction fibers in any motile system would be to polymerize or provide an actin filamentous base which could stick to substrate. The rest of the cell could then move over this more immobile actin base (2D) or through it (3D), perhaps by the action of myosin-like molecules and forward movement of other actin polymer.

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