Role of actin-filament disassembly in lamellipodium protrusion in motile cells revealed using the drug jasplakinolide

Louise P. Cramer

Background: In motile cells, protrusion of the lamellipodium (a type of cell margin) requires assembly of actin monomers into actin filaments at the tip of the lamellipodium. The importance of actin-filament disassembly in this process is less well understood, and is assessed here using the actin drug jasplakinolide, which has two known activities – inhibition of filament disassembly and induction of an increase in actin polymer.

Results: In cells the two activities of jasplakinolide were found to be separable; 1 μM jasplakinolide could permeate cells, bind cellular filamentous actin (F-actin) and inhibit filament disassembly within 3.5 minutes, but significant increase in actin polymer was not detected until 60 minutes of treatment. In live, permeabilised cells, jasplakinolide did not inhibit filament assembly from supplied, purified actin monomers. In migrating chick fibroblasts, lamellipodium protrusion was blocked within 1–5 minutes of treatment with 1 μM jasplakinolide, without any perturbation of actin organisation. In non-migrating chick fibroblasts, there was a delay in the onset of jasplakinolide-induced inhibition of lamellipodium protrusion, during which lamellipodium length increased linearly with no increase in protrusion rate. Motility of the bacterium *Listeria* in infected PtK2 cells was reduced 2.3-fold within 3 minutes of treatment with 1 μM jasplakinolide.

Conclusions: Actin-filament disassembly is tightly coupled to lamellipodium protrusion in migrating chick fibroblasts and motility of *Listeria* in PtK2 cells. One simple interpretation of these data is a situation whereby ongoing actin-filament assembly uses free actin monomer derived from filament disassembly, in preference to stored monomer.

Address: MRC-Laboratory Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT and The Randall Institute, Kings College, London WC2B 5RL, UK.

Correspondence: Louise P. Cramer
E-mail: l.cramer@ucl.ac.uk

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Background

In motile cells, protrusion of the lamellipodium is involved in wound healing and immune cell activation, and is required for entry of certain pathogenic organisms into cells. Protrusion motility is key to bringing the front margin of a migrating cell forward. Protrusion of the lamellipodium (and related structures such as filopodia) is tightly coupled to assembly of actin monomers into actin filaments at the cell margin [1,2]. Protrusion motility has been studied over several decades or so [1–3], but the focus of the work has been on actin-filament assembly and regulation of assembly; filament disassembly has been largely overlooked [2,4].

There must be a constant supply of actin monomers in the region of the cell where a lamellipodium is persistently protruding. Although filament disassembly maintains the general monomer pool, it is not known whether there is a direct link between filament disassembly and protrusion motility. Part of the problem is that it has long been recognised that almost the entire population of cellular actin monomer is stored (not free for assembly) [5], most likely by monomer-sequestering proteins [6] such as thymosin-B4 [7]. If stored monomer is to be used for assembly, it must first be freed from stores, but very little is known about the spatial regulation of this process in the cell. This makes it difficult to predict whether stored actin monomer is generally used during protrusion, and thus difficult to predict the importance of actin filament disassembly in this process. It might turn out that the importance varies in cells, depending on monomer availability. This seems likely as the cellular concentration of actin monomer relative to filaments can vary with cell type and motile cell behaviour. For these reasons, the link between actin filament disassembly and protrusion in cells was tested using jasplakinolide, an inhibitor of actin-filament disassembly, in several different motile cell systems: protrusion of the lamellipodium in migrating cells and in non-migrating cells, and motility of the bacterium *Listeria monocytogenes* [8], a model system for protrusion [9,10] as motility of *L. monocytogenes* is tightly coupled to actin-filament assembly [11,12]. The work also provides new information on actin-assembly dynamics in cells, with implications for regulation of protrusion motility.

A migrating cell is composed of distinct regions: the lamellipodium is at the front of the cell; behind this is the lamella, followed by the cell body, which comprises most of the organelles and the nucleus; at the rear is a rounded or drawn-out tail. The migration process, which results in the entire cell translocating to a new position, comprises protrusion of the lamellipodium, cell-body translocation and tail retraction. Non-migrating cells can also be composed of the same regions and be motile, but only protrusion motility typically occurs with no net cell translocation. A further key difference is that a migrating cell is polarised (typically wedge-shaped or crescent-shaped), with only a single lamellipodium at the front of the cell which persistently protrudes as the cell migrates. In contrast, a non-migrating cell is not polarised (typically orthogonal in shape) and might protrude several lamellipodia at multiple sites around the cell and these tend to persist for shorter periods than migrating cells. Here, lamellipodium protrusion was studied in migrating and non-migrating chick embryo heart fibroblasts, a well-characterised system for investigating motility.

A natural product from a marine sponge [13,14], jasplakinolide specifically targets actin, without affecting microtubules [15], DNA, RNA or protein synthesis [16]. It is similar to the well-known actin inhibitor phalloidin in that, like phalloidin [17,18], jasplakinolide stabilises actin filaments, preventing filament disassembly, both with purified actin [19] and in cells [20]. Like phalloidin [17], jasplakinolide also causes an increase in actin polymer with purified actin [19] and in cells [21,22]. The mechanism underlying this increase has not been reported for jasplakinolide; one possible explanation is spontaneous promotion of actin assembly. Any such activity is expected to be comparatively weak in cells (M. Bubb, personal communication). This is advantageous, allowing one to determine experimental conditions that favour inhibition of filament disassembly without significant increase in actin polymer. Jasplakinolide and phalloidin are the only known inhibitors of actin-filament disassembly but, unlike phalloidin, jasplakinolide is cell-permeant, enabling the effects of blocking actin disassembly in motile cells to be easily studied. Previous work with jasplakinolide has not allowed such an analysis because cells were treated under experimental conditions that caused significant increase in actin polymer and gross disruption of actin organisation. Here, actin-filament disassembly was found to be tightly coupled to protrusion of the lamellipodium during fibroblast migration and *Listeria* motility in PtK2 cells.

Results

Jasplakinolide dose and actin-cytoskeleton organisation

At acute doses, all known actin and tubulin drugs cause general perturbation to their respective cytoskeletons. To allow interpretation of specific effects on cell motility, a dose must first be identified at which general perturbation does not occur. Treatment of migrating and non-migrating chick fibroblasts with 1 μM jasplakinolide for up to 20 minutes, or PtK2 cells with the same dose for up to 15 minutes did not disrupt actin-cytoskeleton organisation nor general cell morphology. Small changes started to occur in 15–30% of fibroblasts at 20 minutes and in PtK2 cells at 15 minutes; small actin filament foci/aggregates appeared, but otherwise the actin cytoskeleton was intact (see below). By 60 minutes of treatment with 1 μM jasplakinolide, the actin cytoskeleton was generally perturbed; large filament aggregates appeared in 100% of fibroblasts and PtK2 cells, with no other actin organisation visible by light microscopy (see below). As motility was studied over a 10 second–1 minute time scale, any specific change in motility could be readily assessed well in advance of general perturbation to actin organisation. Higher doses (5–20 μM) of jasplakinolide induced abnormal actin organisation in 100% of cells by 10 minutes, and are therefore less useful in this context.

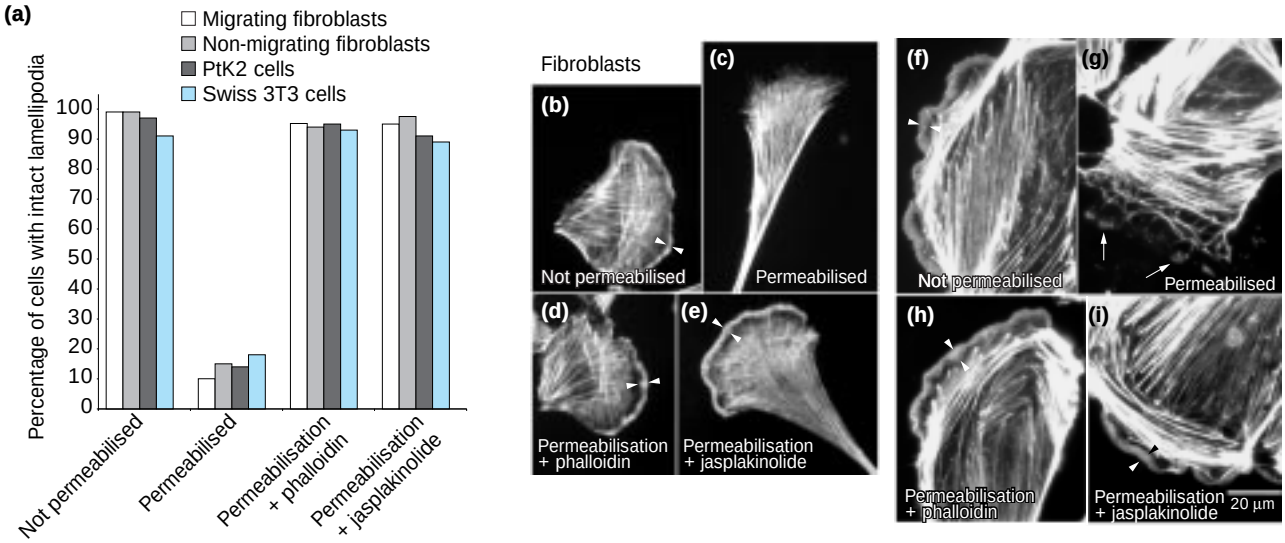
Permeability of cells to jasplakinolide

A test based on competition between jasplakinolide and phalloidin for binding to filamentous actin (F-actin) [19] showed no detectable difference between fibroblasts and PtK2 cells in terms of their permeability to jasplakinolide, and ability of the drug to bind cellular F-actin in the two cell types. Within 2.5 minutes of pretreating live intact cells with 1 μM jasplakinolide, the intensity of Alexa594–phalloidin staining (0.25 μg/ml) of actin filaments in the tip of the lamellipodium in subsequently fixed cells decreased by about one-third compared with non-pretreated cells (decreasing by 35% ± 3% in migrating fibroblasts, 33% ± 5% in non-migrating fibroblasts, and 38% ± 3% in PtK2 cells; average of 28–44 measurements in 20–40 cells). The lamellipodium tip was measured to avoid any potential overestimation of fluorescence intensity in jasplakinolide-treated cells where filament disassembly is blocked; filament disassembly is thought to occur predominantly at the rear, not at the tip of the lamellipodium. A similar decrease in fluorescence intensity was seen in Swiss 3T3 cells, a fibroblast cell line. Thus, it is probable that, in cells in general, entry of jasplakinolide into cells and its binding to cellular F-actin is rapid.

Assessment of jasplakinolide's actin-filament-stabilising and actin-filament-promoting activity

Jasplakinolide (0.55–2.2 μM) inhibits disassembly of preformed actin filaments within 30 seconds of adding the drug in a dilution assay *in vitro* (M. Bubb, personal communication). In contrast, it takes tens of minutes for jasplakinolide to promote filament assembly in non-polymerising buffer [19]. Similarly, for fibroblasts and PtK2 cells, stabilisation of actin filaments by jasplakinolide was similarly rapid, but filament-promoting activity comparatively slow. When live cells are briefly permeabilised in buffer without actin protection, lamellipodia

Figure 1



Actin-filament stabilisation in cells. (a) Quantitation of intact lamellipodia. Live migrating and non-migrating fibroblasts, PtK2 cells and Swiss 3T3 cells were permeabilised for 1 min in the absence or presence of 1 μM jasplakinolide or 1 μM unlabelled phalloidin, then fixed and stained for actin filaments; 200 cells were counted in each category. (b–e) Migrating fibroblasts and (f–i) PtK2 cells that have been (b,f) pretreated live in permeabilisation buffer without detergent, or permeabilised live (c,g) without jasplakinolide or phalloidin, or in the presence of (d,h) phalloidin or (e,i) jasplakinolide. Lamellipodia are denoted between pairs of arrowheads in (b,d,e,f,h,i). The lamellipodium in (c) is not preserved and in (g, arrows) poorly preserved as fragments. In contrast to Figure 2, the permeabilisation buffer did not contain exogenous actin monomer; thus, lamellipodia are not protected when permeabilised live in the absence of jasplakinolide or phalloidin.

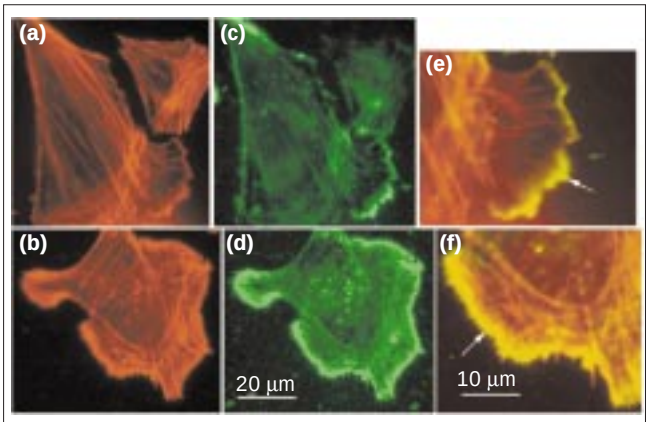
are poorly preserved [23–25] (compare Figure 1b with Figure 1c, and Figure 1f with Figure 1g). This is because of dilution-induced disassembly of actin filaments in lamellipodia. Actin filament bundles in the cell body, having a slower turnover rate, remain intact during brief permeabilisation. Addition of 1 μM jasplakinolide to the permeabilisation buffer prevented filament disassembly in lamellipodia (actin filaments remained intact) within 1 minute in all cell types tested (Figure 1a), as exemplified by migrating fibroblasts (compare figure 1b with figure 1e) and PtK2 cells (compare figure 1f with figure 1i). This effect was indistinguishable from actin protection provided by 1 μM phalloidin in the permeabilisation buffer (compare figures 1d and 1h with figures 1e and 1i). In intact cells, considering the above cell permeability and stabilisation data, jasplakinolide is expected to inhibit actin-filament disassembly within at most 3.5 (2.5 + 1) minutes. That there was no detectable difference in three different cell types suggests that inhibition is rapid in cells in general.

Filament-promoting activity in jasplakinolide-treated cells was measured by immunofluorescence in subsequently fixed fibroblasts and PtK2 cells (see the Materials and methods section). Any increase in actin polymer induced before cell fixation was indicated by an increase in fluorescence intensity. An increase in fluorescence intensity was not detected until 60 minutes treatment with 1 μM jasplakinolide, increasing by 37% in migrating fibroblasts, by 25% in non-migrating fibroblasts, and by 43% in PtK2 cells (average of 10–12 cells measured per cell system). This increase was concomitant with the appearance of large filament aggregates in these cells at 60 minutes (see above and examples below), possibly indicating spontaneous polymer formation. Initiation of filament-promotion might occur before 60 minutes, potentially reflected in the small actin-filament foci that appeared in a small proportion of cells after 15–20 minutes treatment (see above and example below). Whether all aggregates reflect spontaneous polymer formation is unknown. It is plausible that some aggregates form by coalescence of pre-existing actin filaments and bundles, consistent with the absence of filament bundles (see below) around 60 minutes of treatment.

Testing direct inhibition of actin assembly by jasplakinolide

There are no data in the literature to suggest that jasplakinolide directly blocks actin-filament assembly. In live permeabilised fibroblasts, 1 μM jasplakinolide did not inhibit cellular assembly of filaments from supplied exogenous actin monomers (Figure 2). A mixed population of migrating and non-migrating fibroblasts was tested simultaneously (see the Materials and methods section) and no difference in ability to incorporate actin in cells was detected. Also, under the conditions used to study

Figure 2



Incorporation of exogenous actin monomer in cells. **(a,c,e)** Control fibroblasts and **(b,d,f)** fibroblasts treated with 1 μM jasplakinolide were permeabilised live for 10 min in the presence of 1 μM fluorescently labelled exogenous actin monomer, then fixed and stained for total F-actin. (a,b) Total cellular F-actin (red). (c,d) Exogenous actin incorporated into cells (green). (e,f) Twofold magnification of the merged images of (a,c) and (b,d), respectively. The exogenous actin assembled into filaments is highest in lamellipodia (yellow or yellow-green, arrow). The scale bar in (d) applies to (a–d), the scale bar in (f) applies to (e,f).

motility, jasplakinolide (1 μM, up to 20 minutes) did not alter known spatial differences in actin dynamics in cells; most rapid actin dynamics occurred in the lamellipodium (Figure 2e,f; yellow, arrow). In a second test using intact, migrating and non-migrating fibroblasts cultured separately, endogenous actin filaments in lamellipodia were first selectively disassembled (actin filament bundles in the cell body remaining intact) by gentle pretreatment with latrunculin-A (5 μM, 5 minutes). Jasplakinolide (1 μM) did not block reassembly of filaments and reformation of lamellipodia when present during washout of latrunculin-A (data not shown). This also shows that jasplakinolide neither sequesters actin monomer, blocks nucleation of new filament formation, nor interferes with spatial coupling between filament assembly and forward movement of the cell margin.

Considering these data together, a conservative estimate is that any change in behaviour when treating chick fibroblasts for up to 20 minutes and PtK2 cells for up to 15 minutes with 1uM jasplakinolide specifically reflects inhibition of actin-filament disassembly.

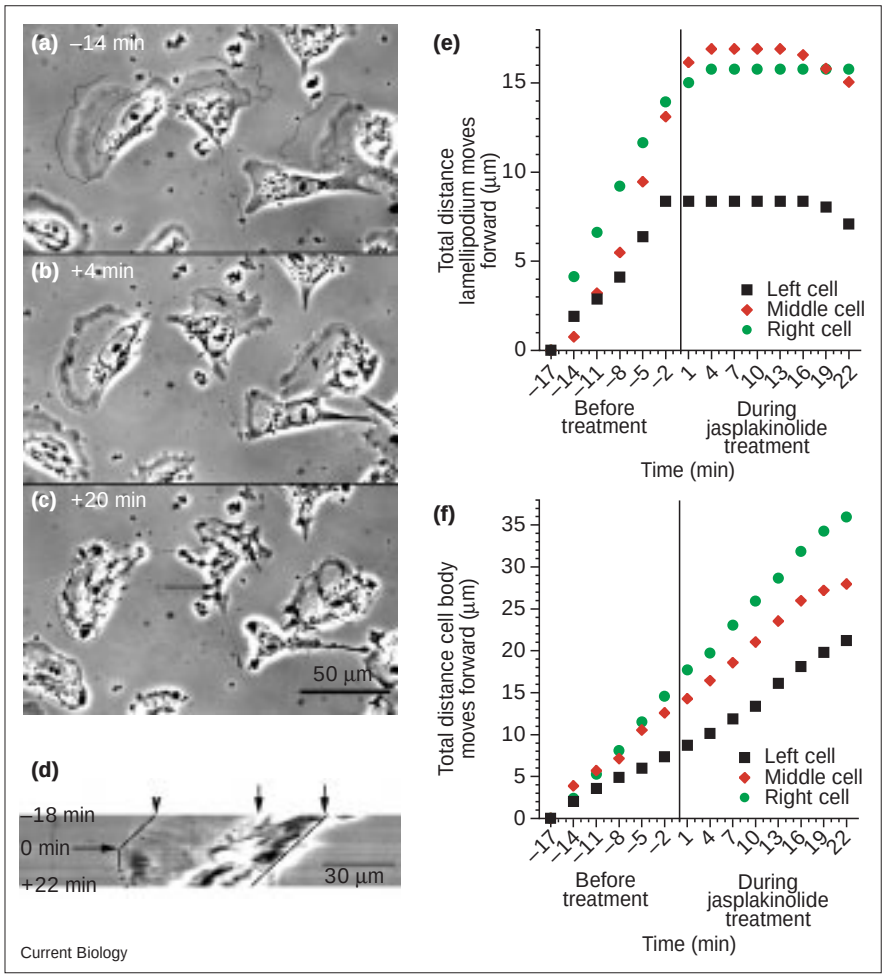
Jasplakinolide rapidly inhibits protrusion of the lamellipodium in migrating fibroblasts

In individual migrating fibroblasts, 1 μM jasplakinolide fully inhibited protrusion of the lamellipodium within 1–5 minutes (Figure 3d, vertical line; Figure 3e), but translocation of the cell body remained constant (Figure 3a–d,f). Inhibition of protrusion also occurred at 200–500 nM jasplakinolide, but was less effective at 100 nM (data not shown). In the cell population, inhibition of protrusion averaged 3–5 minutes (see below). Once protrusion was inhibited, then over the next 5–10 minutes of treatment, the lamellipodium typically stayed 'frozen' in place (Figure 3d, vertical line), or slightly retracted in discrete zones. Also, once protrusion was inhibited, new lamellipodia did not form at new cellular sites. By 20 minutes of treatment, the lamellipodium had typically retracted, either completely or in larger discrete zones (Figure 3c,d; diagonal dots). Because the cell body persisted in moving forward during the entire course of jasplakinolide treatment (10–30 minutes for individual experiments), it encroached on the cell margin (clearly seen in Figure 3d). As expected, a similar effect occurs when migrating keratocytes are treated in the short term with cytochalasin [26].

Although cell-body translocation in migrating fibroblasts persisted in jasplakinolide, there was an effect on the direction of motility. In all jasplakinolide-treated cells, the cell body continued to move forward (towards the lamellipodium), as occurs normally during cell migration but, in around 70% (14/20) of the cells, small portions of the cell body lost coherence and broke away, moving instead towards the rear (for example, Figure 3c, middle cell between long arrow and arrowhead) or side of the cell. One hypothesis is that ongoing protrusion simply plays a role in maintaining coherent forward motion of the cell body.

Inhibition of protrusion by 1 μM jasplakinolide was not because of potential general disruption of the actin cytoskeleton, nor general inactivation of actin-based motility. At the time protrusion was inhibited (1–5 minutes), cell morphology (clearly observed in the movie published as Supplementary material with this article on the internet, and in Figure 3b) and actin organisation (compare Figure 4a with Figure 4b) were normal at the level of resolution of the light microscope. Further cell-body translocation in treated cells was not inhibited (see above). Translocation of the cell body in these cells is driven by myosin interacting with already assembled actin filament bundles [27]. As these bundles remained intact when translocation was occurring in the presence of jasplakinolide (for example, Figure 4c, long arrow), stabilisation of actin filaments by jasplakinolide *per se* does not prevent actin-based motility. As a further control, another type of myosin-driven cell motility, cell respreading after mitosis in PtK2 cells [24,28], was also not inhibited by 1 μM jasplakinolide (Table 1). Higher concentrations of jasplakinolide (up to 2–3 μM), 15-times higher than the lowest concentration that inhibits protrusion in fibroblasts, also did not inhibit cell respreading after mitosis.

Figure 3



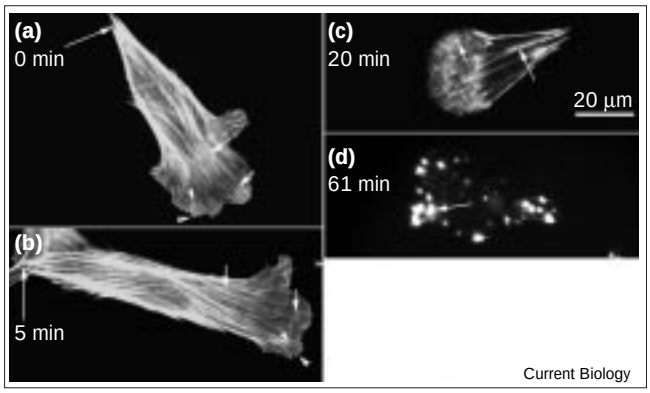
Jasplakinolide treatment of migrating fibroblasts. Live migrating fibroblasts were treated with 1 μM jasplakinolide and imaged every minute by phase contrast time-lapse microscopy. **(a–c)** The three pairs of outlines indicate the position to which three migrating cells had moved to after 4 min in jasplakinolide (b). The outlines correspond to the position of the margin of the lamellipodium and front boundary of the cell body in (b). (a) Control, 14 min before addition of jasplakinolide. (b) After 4 min in jasplakinolide. The lamellipodium did not protrude beyond the longer outline from its position in (a; arrowhead). In the left cell, protrusion stopped by 1 min (see (d)), and in the other cells by 3–4 min (see (e)). The cell body moved from its position in (a; arrow) to the shorter outline. (c) After 20 min in jasplakinolide. The lamellipodium retracted (gap between cell edge and longer outline). The cell body continued moving forward from its position in (b; from the shorter outline to the short arrows). The overall appearance of the cell body was less coherent. A cell-body fragment moved to the rear cell margin (middle cell; from the long arrow, which indicates the position at 10 min in jasplakinolide, to the arrowhead). **(d)** Kymograph image (time–position plot) along a line (0.7 μm wide) of the vector of migration, perpendicular to the lamellipodium, of the left cell in (a–c). The cell is migrating towards the left. Cell position (horizontal axis) on the line is shown every minute (vertical axis) from 18 min before addition of jasplakinolide (0 min, long arrow) to 22 min after addition. The lamellipodium is uniform grey density on the left (for example, arrowhead). The cell body is a mixture of light and dark phase density on the right (for example, between the arrows). Diagonal solid lines towards the left are examples of constant forward motility. The vertical line indicates inhibition of protrusion. Diagonal dots towards the right indicate retraction of the lamellipodium. **(e,f)** History of (e) lamellipodium protrusion and (f) cell-body translocation before and during jasplakinolide treatment for the three migrating cells shown in (a–c). The scale bar in (c) applies to (a–c).

Effect of jasplakinolide on lamellipodium protrusion in non-migrating fibroblasts

Although jasplakinolide started to inhibit protrusion in migrating fibroblasts within 1–2 minutes (Figure 3e), the onset of inhibition was delayed by about 10 minutes in non-migrating fibroblasts (Figure 5a, filled symbols). Protrusion rate then slowed down, with full inhibition within 12–20 minutes in individual non-migrating fibroblasts (Figure 5a, filled symbols; Figure 6, squares). This difference was not simply because there is more F-actin to stabilise in non-migrating cells; in untreated cells, F-actin was 1.3-fold higher in lamellipodia, and 1.1-fold higher in the rest of the cell, yet overall it took fourfold longer to completely inhibit protrusion. Also, it was neither because of a difference in the rate of protrusion of the lamellipodium (Table 1, before treatment, compare non-migrating with migrating cells), nor to general disruption of the actin cytoskeleton when protrusion is inhibited (compare Figure 5c and Figure 5d).

In non-migrating fibroblasts, as was expected, when disassembly, but not lamellipodium protrusion, was inhibited, the lamellipodium increased in length (in the direction of the protrusion). This was a specific effect and was not because of an increase in protrusion rate; as mentioned above, this increase did not occur. Increase in lamellipodium length was readily detected (see Materials and methods) in individual live cells from the time-lapse record (Figure 5a, open symbols) and in fixed and stained cells (Figure 5b; Figure 5f, compare upper and lower images, between pairs of vertical lines). In live cells, the length of a given lamellipodium was constant before jasplakinolide treatment, but increased linearly with time in the presence of the drug (Figure 5a, open symbols). Each

Figure 4



Actin-filament staining in jasplakinolide-treated migrating fibroblasts. Cells were (a) untreated or (b–d) treated live with 1 μM jasplakinolide for 5, 20 and 61 min, respectively, then fixed, permeabilised and stained for actin filaments. In (a,b), the lamellipodium is denoted between pairs of arrowheads; the lamella, behind the lamellipodium, is located between the short arrows; the cell body and rear, behind the lamella, are located between the innermost short arrow and the long arrow. In (b), note that the actin cytoskeleton is not perturbed and the lamellipodium remains intact. In (c), the lamellipodium has retracted (as expected, see Figure 3). In 15–30% of cells, small actin filament foci have appeared in the lamella (short arrow); actin bundles in the cell body remained intact (long arrow). In (d), the actin cytoskeleton is perturbed; actin filaments are aggregated (arrow). The scale bar in (c) applies to (a–d).

lamellipodium stopped increasing in length (Figure 5a, open symbols, plateaus on the plots) concomitant with inhibition of protrusion in the same cell (Figure 5a, filled symbols, plateaus on the plots). As expected, when individual lamellipodia in untreated cells were compared, there was a range of lengths (Figure 5b, unshaded bars). Thus, not unexpectedly, this also occurs in jasplakinolide-treated cells, but the range was clearly shifted to longer lengths (Figure 5b, compare unshaded and shaded bars). Comparing lamellipodia at 10 and 20 minutes treatment, the length distribution overlapped (Figure 5b, compare shaded bars). This was highly consistent with inhibition of protrusion (12–20 minutes, see above). By 20 minutes of jasplakinolide treatment, lamellipodia had on average increased 6.2-fold (7.3/1.1 μm) in length, an average length increase of 6.2 μm, observed for both live and fixed cells. This was reasonably close to the increase in length (8.7 μm) estimated from the average rate of protrusion in treated cells (Table 1). The difference is likely a consequence of variation in the time it took for individual lamellipodia to stop protruding.

Also as expected, when actin-filament disassembly was inhibited, filaments apparently accumulated towards the back of the lamellipodium (Figure 5f, arrows). Such an accumulation was not observed in untreated cells, and was clearly distinct from dorsal actin ruffles which, in these cells, are characteristically thin and wavy. Also in a small proportion of cells slight promotion of filament assembly (see above) might make a minor contribution to filament accumulation. In migrating cells, jasplakinolide also induced an increase in lamellipodium length by an average 2.2 μm, before protrusion was blocked within 5 minutes. In contrast to non-migrating cells, an accumulation of actin filaments was not detected at the rear of the lamellipodium; presumably, comparatively little actin accumulates before rapid inhibition of protrusion in migrating cells.

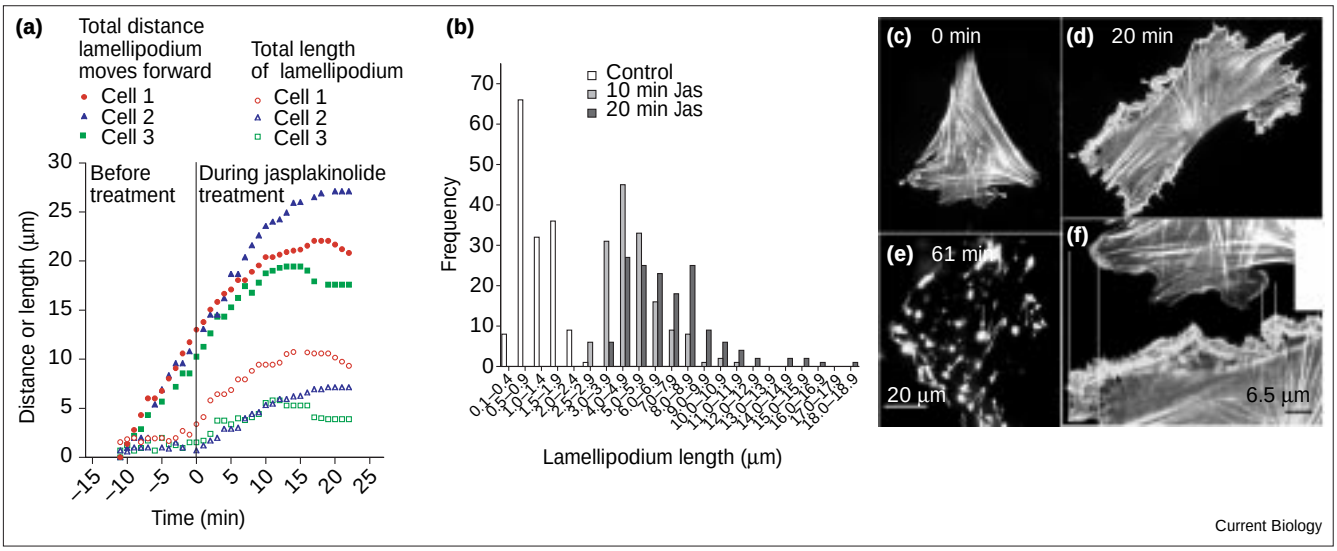
Effect of jasplakinolide on *Listeria* motility

The initial response of *Listeria* motility in jasplakinolide-treated PtK2 cells was very similar to protrusion in migrating fibroblasts. For both systems, there was a rapid decrease in the rate of motility, on average to less than one half of the control by 3 minutes of treatment with jasplakinolide (Figure 6, compare circles and diamonds; Table 1). Longer exposure to jasplakinolide revealed a difference; it took on average 3–5 minutes to completely inhibit protrusion in migrating fibroblasts, but 3–15 minutes to completely inhibit all *Listeria* motility (Figure 6, compare circles and diamonds). It was not possible to determine from the time-lapse record (phase-contrast images) whether *Listeria* tails increased in length in treated cells, as only the bacterium body was highly visible by this method.

Discussion

This study revealed that short-term treatment with jasplakinolide caused immediate inhibition of protrusion in migrating fibroblasts; inhibited lamellipodium protrusion in non-migrating fibroblasts but with a delay in the onset; and rapidly reduced the rate of *Listeria* motility in Ptk2 cells. In each of these three systems, cellular response to jasplakinolide (Figure 3e; Figure 5a, open symbols; Figure 6, circles) correlated temporally with inhibition of actin-filament disassembly but not with promotion of an increase in actin polymer, a weaker activity of jasplakinolide. In the absence of any other known jasplakinolide activity in cells, this argues strongly that lamellipodium protrusion and *Listeria* motility (and, thus, actin-filament assembly) are coupled to filament disassembly. The data provide direct evidence for previous arguments in the literature [4]. In the lamellipodium of migrating fibroblasts (for example Figure 6, diamonds) and with *Listeria* motility in PtK2 cells (Figure 6, circles), the coupling was fast, suggesting that filament disassembly is rate limiting for protrusion and *Listeria* motility in these cell systems. In contrast, in lamellipodia of non/slowly-migrating fibroblasts the coupling was slower (for example Figure 6, squares), suggesting a distinct rate-limiting step. How is this explained? Because detailed characterisation of biochemical properties of jasplakinolide cannot account for slower coupling, the most likely explanation is a difference in the actin cycle. A simple theory that I favour is that fast coupling reflects a preference for disassembling

Figure 5



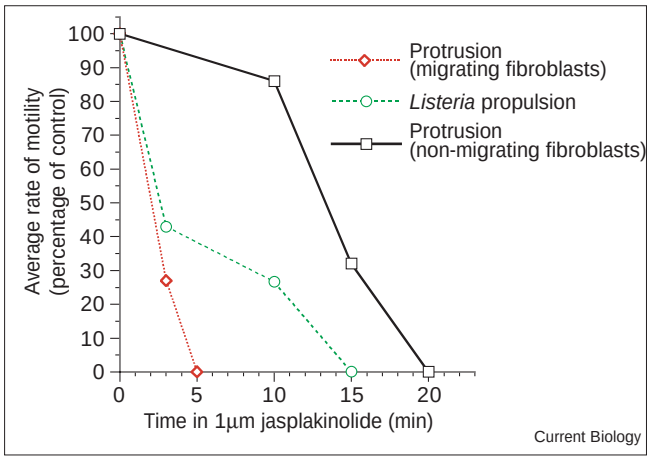
Jasplakinolide treatment of non-migrating fibroblasts. Live non-migrating fibroblasts were treated with 1 μM jasplakinolide and (a) imaged every minute by phase contrast time-lapse microscopy, or (b–f) pretreated live then fixed, permeabilised and stained for actin filaments. (a) History of lamellipodium protrusion (filled symbols) and lamellipodium length (in the direction of protrusion, open symbols) for three non-migrating live cells. (b) Lamellipodium length distribution in fixed cells. In each case, 154 measurements of 48–52 lamellipodia were made in 25–26 cells. The average length was 1.1 ± 0.04 μm for untreated cells, 5.1 ± 0.13 μm and 7.3 ± 0.22 μm for cells treated with jasplakinolide for 10 min and 20 min, respectively. (c) Untreated cell. (d,e) Cells treated with 1 μM jasplakinolide for (d) 20 min or (e) 61 min. Examples of lamellipodia are indicated between pairs of arrowheads in (c,d) or pairs of lines in (f). In (d), the actin cytoskeleton is not perturbed and lamellipodia remain intact; in some cells, lamellipodium retraction began to occur around 20 min (see (a); filled symbols). In (e), the actin cytoskeleton is perturbed; actin filaments are aggregated (arrow). (f) The upper and lower images are twofold magnifications of lamellipodia shown in (c,d), respectively. The lower image has been rotated roughly 30° clockwise for comparison with the upper image. Note apparent accumulation of actin filaments at the back of the lamellipodium in the treated cell (arrows). The scale bar in (e) applies to (c–e). The scale bar in (f) applies to (f).

filaments as the source of required free monomer for filament assembly (Figure 7a, fast-coupled model). Conversely, in slow coupling, stored monomer is the preferred source (Figure 7b, slow-coupled model); in this model, filament disassembly instead refuels stored monomer. This is consistent with the initial 10 minute lag in inhibition of protrusion when disassembly is blocked with jasplakinolide in non-migrating cells (stored monomer is depleted), followed by a decrease in the rate of protrusion (depleted stores are not replaced). These simple scenarios invoke a direct role for monomer desequestration in slow coupling, but not fast coupling.

A more complex theory is that the buffering capacity (balance between sequestration and desequestration) thought to exist for certain sequestering proteins [29] is coupled to filament disassembly in both fast coupling and slow coupling. In fast coupling, free monomer made available by filament disassembly is immediately sequestered, rapidly promoting desequestration elsewhere in the lamellipodium. In slow coupling, buffering must be leaky or less rapidly coupled to filament disassembly. Another interpretation of the data is that blocking disassembly inhibits feedback regulatory systems required for protrusion. This imposes differential regulation of protrusion in migrating and non-migrating fibroblasts. Distinguishing between these models will require direct observation of the origin and fate of free monomer in live cells as it becomes available in the lamellipodium, an investigation beyond the scope of this study. Whether the situation in migrating and non-migrating fibroblasts reflects the situation for migrating and non-migrating cell types in general remains to be tested. One possibility is that the mechanism in any given cell type is instead determined by the relative concentrations of actin filaments and actin monomer, for example.

Although fast-coupling occurs for both protrusion in migrating fibroblasts and *Listeria* motility, there is a subtle difference between the two motile cell systems. This is that it takes a relatively long time to completely inhibit all *Listeria* motility (Figure 6, compare circles and diamonds). This is difficult to interpret without further experimentation. One intriguing possibility is explained by location-dependent accessibility of actin monomer. The bulk of *Listeria* move in the cell body and lamella of cells and, in these locations in PtK2 cells, there is a sizeable pool of actin monomer (unpublished observations). *Listeria* may also be capable of

Figure 6



Comparison of lamellipodia protrusion and *Listeria* motility in jasplakinolide. Migrating and non-migrating fibroblasts, and PtK2 cells infected with *Listeria*, were treated with 1 µM jasplakinolide. Absolute rates of motility were measured for individual live cells for each cell system, then the population average calculated (Table 1) and expressed as a percentage of the control (time = 0 min). For lamellipodium protrusion, rates were calculated between each successive time interval plotted on the graph; the control was determined by measuring protrusion over the preceding 5–10 min before addition of jasplakinolide. For *Listeria* motility, rates were determined over 30–60 sec at the indicated times in jasplakinolide.

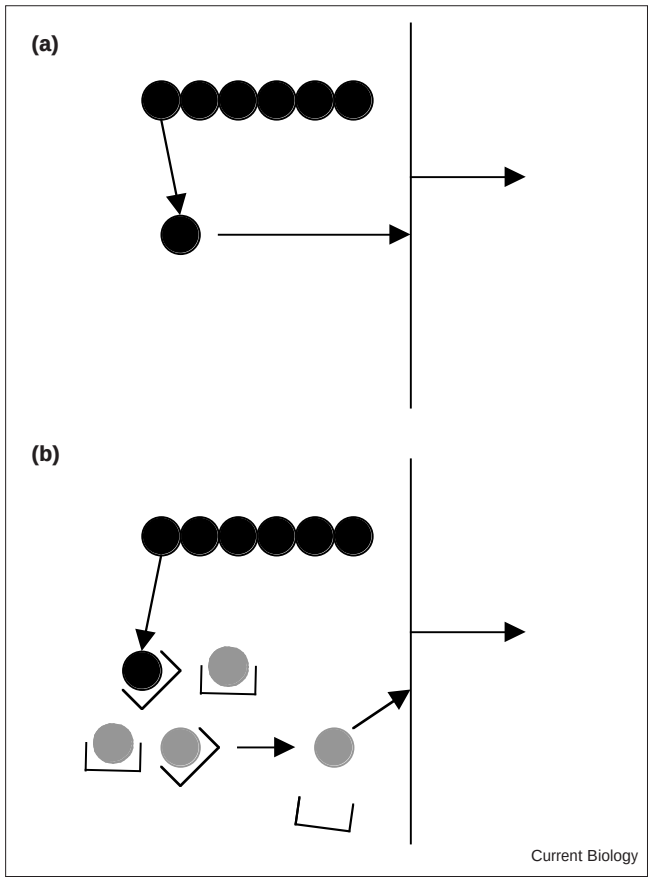
consuming this monomer pool during motility. Previous work in cell extracts has suggested an important role for actin-depolymerising factor (ADF)/cofilin in disassembly of actin filaments in *Listeria* tails [30,31]. Here, the data on jasplakinolide effects in intact cells reveal significant dependence on filament disassembly for *Listeria* motility.

Why actin-filament disassembly is more important for protrusion in certain cell systems, and how cells harness disassembling filaments for motility is unknown. That this occurs in cells reveals an interesting and previously unrecognised aspect of actin-assembly dynamics. It may turn out that the importance of filament disassembly for motility in an individual cell type is dependent on monomer availability and cell activity. Stored actin monomer allows a large stockpile of monomer to accumulate in an inactive cell. This is ideal for responding rapidly to an initial signal that initiates motility by freeing monomer for the first round(s) of assembly. This is the situation when cell protrusion is initially activated in resting platelets [32], leucocytes [33] and thyone sperm [34,35]. For an already active cell (such as a migrating fibroblast), ongoing lamellipodium protrusion might instead be maintained by cycles of assembly and disassembly.

Conclusions

Jasplakinolide joins the growing list of small-molecule inhibitors of the actin cytoskeleton. When used in the

Figure 7



Coupling between actin-filament disassembly and protrusion of the lamellipodium. In theory, there are two extreme roles for actin-filament disassembly during protrusion of the lamellipodium. In both, protrusion is tightly coupled to actin-filament assembly, but the rate of protrusion is limited by filament disassembly in the (a) fast-coupled, but not the (b) slow-coupled model. (a) Fast-coupled model. Disassembly directly provides free monomers (black circles) needed for incorporation into filaments at the leading cell margin. No direct evidence exists for such a mechanism, but the rate of filament disassembly is fast enough to account for known rates of protrusion [4]. (b) Slow-coupled model. Disassembly simply refuels (black circle in bracket) stored monomer (grey circles in brackets). Protrusion requires release of monomer from these stores (grey circle freed from bracket). There is some evidence for this mechanism in the literature [32–34].

short term in cells, it is a useful tool to study actin-filament disassembly, without perturbation of cellular actin organisation. Longer-term treatment promotes appearance of filament aggregates, and gross disruption to actin organisation. The precise time that this occurs shortens with increasing jasplakinolide concentration, and the drug should be titrated in any given cell system of interest. This study reveals rapid coupling between actin-filament disassembly and protrusion motility. Whether this implicates an important role for filament disassembly in supplying the lamellipodium with a direct source of free actin monomer remains to be tested. This will not be established until

Table 1

Effect of 1 μM jasplakinolide on actin-based cell motility.

Type of cell motility	Average rate of motility*			Inhibition of motility (min†)
	Before treatment	Jasplakinolide treatment (3 min)	Jasplakinolide treatment (10 min)	
Lamellipodium protrusion (migrating fibroblasts)	0.89 ± 0.06 (*n* = 20)	0.24 ± 0.03 (*n* = 20)	NA	1‡–5
Listeria motility (in PtK2 cells)	10.1 ± 0.6 (*n* = 30)	4.32 ± 0.5 (*n* = 28)	2.65 ± 0.6 (*n* = 16)	3‡–15
Lamellipodium protrusion (non-migrating fibroblasts)	0.87 ± 0.04 (*n* = 22)	ND	0.75 ± 0.04 (*n* = 22)	12–20
Cell-body translocation (migrating fibroblasts)	0.81 ± 0.06 (*n* = 17)	ND	0.79 ± 0.05 (*n* = 17)	Not inhibited
Cell respreading after mitosis (PtK2 cells)	1.08 ± 0.13 (*n* = 10)	ND	0.98 ± 0.08 (*n* = 10)	Not inhibited

*For lamellipodium protrusion, cell-body translocation and cell respreading after mitosis, rates (μm/min) were determined over the total time indicated in jasplakinolide and typically over 5–10 min before treatment. Over this time interval before treatment, motility is generally continuous and thus rates, when compared, are not overestimated or underestimated. For *Listeria*, which move at much higher rates and typically the same bacterium can be identified and tracked in the cell for around 1 min, rates were measured over 30–60 sec before treatment and over 30–60 sec at each specified total time in jasplakinolide. NA, not applicable; ND, not determined. †For fibroblasts this is for individual cells, for *Listeria* this is the range over which motility rate is reduced; it is not possible to track individual *Listeria* over the entire course of drug treatment. ‡ This is the earliest time in drug treatment that the cells were imaged by timelapse microscopy.

spatial and temporal differences in control of actin monomer and filament pools are examined. In the long term, these analyses will contribute to the understanding of the mechanism of protrusion, cell migration and *Listeria* motility. That filament disassembly is rate limiting for protrusion of the lamellipodium in migrating chick fibroblasts means that regulation of filament disassembly is critical for protrusion in this migrating cell type.

Materials and methods

Preparation of primary chick fibroblasts, and PtK2 cell culture

To obtain individual migrating fibroblasts, explant heart tissue from 6–7 day old chick embryos was gently pipetted under media onto glass coverslips coated with matrigel (Becton Dickson) and poly-L-lysine (Sigma). Under these conditions, there is first a lag phase (12–24 h) during which the explant attaches to the matrigel/poly-L-lysine and cells migrate to the boundary of the explant itself. The first fibroblasts to reach the boundary of the explant were used for experiments within 0–12 h (12–36 h total from plating the explant). These are a population of rapidly migrating fibroblasts with characteristic polarised morphology and speed of migration (0.5–1.5 μm/min). The variation in the lag phase was recently discovered and varies with batch of matrigel from the supplier. The lag time makes no difference to the behaviour of cells (in terms of polarised morphology, organisation of the actin cytoskeleton, behaviour in jasplakinolide) once they have cleared the boundary of the explant. Non-migrating or slowly migrating chick heart fibroblasts were prepared as above, but cultured for 4–8 days. It has long been recognised that primary fibroblasts lose their characteristic polarised morphology and ability to migrate rapidly with longer time in culture [36]. For the filament-assembly test (Figure 2), a mixed population of migrating and non-migrating fibroblasts on the same individual coverslip was prepared by culturing cells from the same explant preparation for 2–3 days. PtK2 cells were grown [28] and infected with *L. monocytogenes* [37] essentially as described. In general and particularly for the actin-disassembly test (Figure 1), PtK2 cells were cultured at lower density to promote growth as individual cells or cells in islands with lamellipodia. When cultured at higher density, PtK2 cells grow in monolayers or sheets and do not generally protrude lamellipodia.

Drug treatment of cells

Live cells were treated with 1 μM jasplakinolide or 5 μM latrunculin-A, made-up in warm media immediately before use from a frozen 500–1000 × DMSO stock. Final DMSO added to cells was 0.1–0.2%, which alone has no effect on cell behaviour or organisation of the actin cytoskeleton (data not shown). For time-lapse experiments on fibroblasts and cell respreading after mitosis in PtK2 cells, jasplakinolide was added to cells directly on the microscope after the pre-treatment sequence was recorded, without stopping the time-lapse acquisition. For cell respreading after mitosis, jasplakinolide was added towards the beginning of respreading. This allowed maximal treatment with jasplakinolide before cells reached the end of respreading. For experiments on PtK2 cells infected with *Listeria*, cells were time-lapsed by Lisa Belmont (University of California, Berkeley) at periodic intervals before and during treatment with 1 μM jasplakinolide.

Cell staining

Unless stated otherwise, cells were stained for actin filaments with Alexa594–phalloidin (Molecular Probes) at 0.25–1 μg/ml. In brief, cells were fixed in 4% formaldehyde in cytoskeleton buffer, permeabilised in 0.5% Triton-X-100, and blocked in 2% BSA, using a method that optimally preserves the actin cytoskeleton [24]. For the filament-disassembly test (Figure 1), live cells were permeabilised in cytoskeleton buffer plus 0.1% Triton-X-100. For incorporation of exogenous actin in live permeabilised cells (Figure 2), the method of Symons and Mitchison [23] was followed, except live cells were permeabilised with 0.4 mg/ml saponin for 10 min in the presence of Alexa488–rabbit skeletal muscle actin monomer (Molecular Probes).

To measure cellular actin polymer, cells were fixed for 45–60 sec in –20°C methanol, after pretreating them live for 0, 5, 20 and 60 min with 1 μM jasplakinolide. Actin filaments were then visualised by indirect immunofluorescence, using a monoclonal anti-peptide antibody to

actin (Sigma). Only actin filaments are stained under the methanol fixation conditions for this antibody. Brief methanol fixation results in good preservation of the actin cytoskeleton.

Image acquisition

For time-lapse microscopy, cells were transferred on the day of the experiment to a custom-made aluminium chamber heated by a circulating waterbath set at 37°C (final temperature in the media in the chamber was around 34°C). Time-lapse microscopy of live cells and taking still images of fixed and stained cells were performed using a cooled charge-coupled device camera (KAF 1400, Princeton Instruments) on an Axiovert microscope (Zeiss) controlled by Metamorph software (Universal Imaging).

Image processing, lamellipodium length measurement and fluorescence quantitation

Images were digitally captured and processed. Merged images (Figure 2e,f), the kymograph (Figure 3d), lamellipodium measurements, and fluorescence quantitation were done with Metamorph software (Universal Imaging). The camera has a small pixel size (6.9 μm), large pixel array (1317 × 1075), and is 12-bit with > 99.9% linearity. This enabled high-resolution images of lamellipodia and highly sensitive fluorescence detection. So that a large sample size could be obtained in a reasonable time, lamellipodium length was measured mainly from fixed cells, stained for actin filaments, captured with a 63 ×, 1.4 NA oil objective. Lengths were also independently measured from phase-contrast images of live cells captured with a 40 ×, 0.75 NA dry objective, and gave the same results. In both cases, individual images were enlarged 200–400% on screen to allow accurate measurement with the Metamorph line-measuring tool. The lamellipodium was readily identified in the cell especially at the high resolution used for measurements. By F-actin staining, the lamellipodium is an intense band, clearly distinguished from the lamella (situated behind the lamellipodium), which is relatively dim. By phase contrast, the boundary between the lamellipodium and lamella is clearly defined; the lamellipodium is thinner, and the lamella is more phase dense and granular in appearance.

For fluorescence quantitation, staining conditions, the microscope objective (63 ×, 1.4 NA oil) and camera exposures in any given cell system and when comparing cell regions in migrating and non-migrating fibroblasts, were the same. All measurements were made on unprocessed images. Background fluorescence was subtracted from all measurements. In each individual cell or cell region, total fluorescence (sum of all grey levels) was measured. So that cells or cell regions of different sizes could be compared, total fluorescence was normalised to total area. The population average was then calculated. For measuring cellular actin polymer in jasplakinolide, the relative percentage increase in fluorescence (comparing before and after treatment) was then determined.

Supplementary material

Movies are available at http://current-biology.com/supmat/supmatin.htm.

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