Enforced polarisation and locomotion of fibroblasts lacking microtubules

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The polarisation and locomotion of fibroblasts requires an intact microtubule cytoskeleton [1]. This has been attributed to an influence of microtubule-mediated signals on actin cytoskeleton dynamics, either through the generation of active Rac to promote protrusion of lamellipodia [2], or through the modulation of substrate adhesion via microtubule targeting events [3,4]. We show here that the polarising role of microtubules can be mimicked by externally imposing an asymmetric gradient of contractility by local application of the contractility inhibitor ML-7. Apolar fibroblasts lacking microtubules could be induced to polarise and to move by application of ML-7 by micropipette to one side of the cell and then to the trailing vertices that developed. The release and retraction of trailing adhesions could be correlated with a relaxation of traction on the substrate and a differential shortening of stress-fibre bundles, with their distal tips relaxed. Although retraction and protrusion in these conditions resembled control cell locomotion, the normal turnover of adhesion sites that form behind the protruding cell front was blocked. These findings show that microtubules are dispensable for fibroblast protrusion, but are required for the turnover of substrate adhesions that normally occurs during cell locomotion. We conclude that regional contractility is modulated by the interfacing of microtubule-linked events with focal adhesions and that microtubules determine cell polarity via this route.

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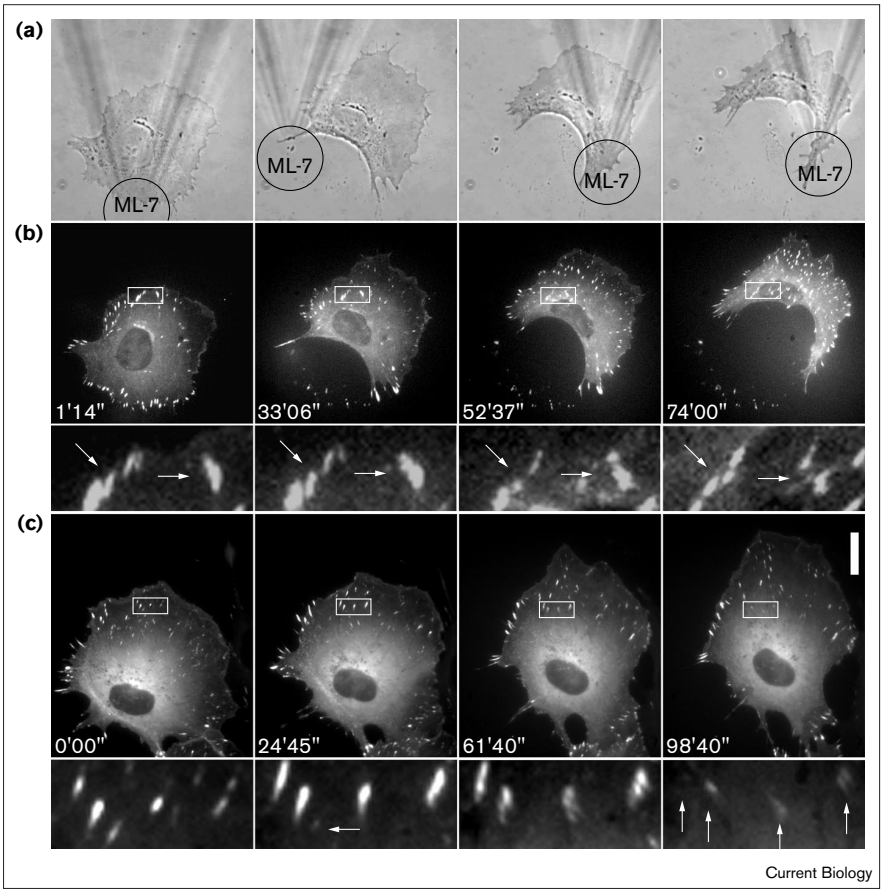
Results and discussion

It has been demonstrated recently [4] that the focal application of the myosin light-chain kinase inhibitor ML-7 to the edge of a spread fibroblast causes the dissociation of peripheral adhesions and local retraction of the cell edge. We also noted that continued application of ML-7 at the retracting edge caused protrusion of the cell on the opposite side. We have now extended these findings to fibroblasts in which the microtubules were depolymerised by nocodazole and which were in consequence incapable of polarisation and net movement. As shown in Figure 1a (see also Supplementary material), cells lacking microtubules could be induced to polarise and move by focal application of ML-7. Application of buffer alone had no effect (data not shown). The initial asymmetry induced by local application of ML-7 could be propagated by applying the inhibitor to the trailing cell tails that subsequently developed. The result of enhancing retraction on one side of the cell was to enhance protrusion on the other, producing a motile phenotype. By following the cell with the application micropipette, polarised movement of up to several cell lengths could be achieved. Enforced polarisation could not only be induced in fish fibroblasts lacking microtubules (Figure 1a), but also in chick embryo fibroblasts, mouse NIH-3T3 cells and B16 melanoma cells lacking microtubules (data not shown). In the absence of microtubules, the actin cytoskeleton of cells undergoing enforced motility exhibited a typical lamellipodium at the advancing cell front (see Supplementary material).

In normal fibroblasts undergoing directed locomotion, adhesion sites that are formed close to the cell front are characteristically disassembled as they approach the perinuclear region [5]. A typical example of this process is shown in Figure 1c in a normal migrating cell expressing the adhesion site component zyxin tagged with green fluorescent protein (GFP). Figure 1b shows the corresponding situation for the nocodazole-treated cell in Figure 1a undergoing induced polarisation and movement. Adhesion sites developed normally but were not subsequently dissociated, even when the cell body passed over them; they persisted until they reached the cell rear (for example the contact sites indicated by the arrows in Figure 1b). The absence of a dynamic turnover of adhesion sites during enforced polarisation was matched by a retarded turnover of the anterior actin cytoskeleton. In control cells, stress-fibre bundles marked by α-actinin–GFP or actin–GFP were formed behind the advancing cell edge and reorganised as protrusion progressed. This was not the case for nocodazole-treated cells undergoing forced polarisation (see Supplementary material). Instead, protrusion was associated with a lack of turnover of stress fibres that had existed at the designated cell front. In contrast, stress

Figure 1



(a,b) Enforced polarisation and motility of a goldfish fibroblast lacking microtubules. (a) Phase-contrast video frames of a goldfish fibroblast that was preincubated and maintained in nocodazole (2.5 μg/ml) and then treated with ML-7 from the tip of a micropipette (the out-of-focus chevron). Times are in min and sec. The inhibitor was applied to the cell edge with the flow from the pipette directed away from the cell. (b) Fluorescence images of the same cell showing substrate adhesions marked with zyxin tagged with enhanced GFP (zyxin–EGFP). The boxed areas in the upper panels of (b,c) are shown enlarged in the lower panels. Substrate contacts (arrows in lower panels) that formed at the cell front persisted until they reached the cell rear. **(c)** Migrating control fish fibroblast transfected with zyxin–EGFP. Focal contacts that formed at the cell front dissociated as they approached the perinuclear zone (typical examples are indicated by the arrows in the lower panels). Scale bar represents 20 μm. See Supplementary material for movies.

fibres at the rear shortened and subsequently dissolved as the cell tail retracted in response to ML-7.

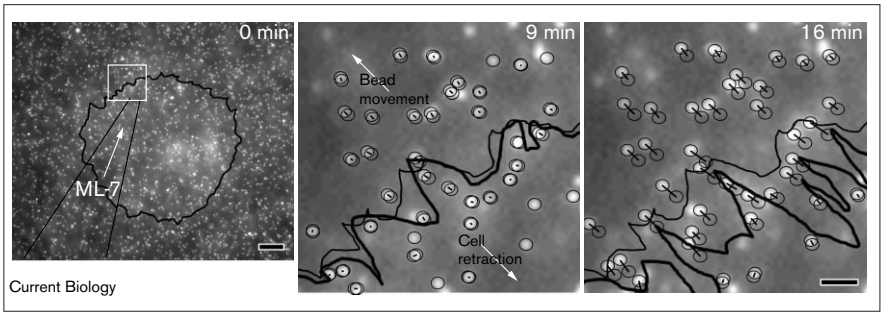
Because the cell periphery was drawn inwards on application of ML-7, we sought to establish if this was associated with a transient increase or decrease in tractional forces on the substrate. This was tested using a growth support of flexible polyacrylamide films impregnated with fluorescent beads [6]. Cultured cells normally exert centripetal forces on the substrate, causing the beads to move toward the cell centre. As shown in Figure 2, ML-7-induced retraction was accompanied by substrate relaxation, indicated by the movements of beads away from the centre of the cell; there was no transient increase in traction. The substrate-relaxation effects seen with ML-7 were similar in type and magnitude to those observed at the tail end of control cells, as reported by Pelham and Wang [6].

In a second set of experiments, the retraction event was followed in cells microinjected with fluorescent smooth muscle myosin (Figure 3). The results revealed that ML-7-induced retraction involved the differential shortening of stress fibre bundles along their length. Thus, active shortening, as evidenced by the concentration of myosin assemblies, occurred in the proximal zones of the stress fibres, whereas the distal zones, close to the site of application, remained more or less unshortened (Figure 3a). We also observed the same general phenomenon in control cells, in which the retraction of a cell vertex during normal locomotion was studied (Figure 3b). We conclude that contact release under these conditions is associated with the relaxation of tension in the region of the substrate adhesion sites.

We show here that fibroblasts lacking microtubules can be forced to polarise and move by the asymmetric application of an inhibitor of contractility. Other agents, such as GRGDS peptides, which mimic the binding site for extracellular matrix in integrins [7,8], caused local contact release and the corresponding extension of the opposite edge. These effects were, however, mild and an order of magnitude slower than those induced by ML-7 (see Supplementary material).

Our results have two implications for the role of microtubules in directed cell locomotion. First, they show that

Figure 2



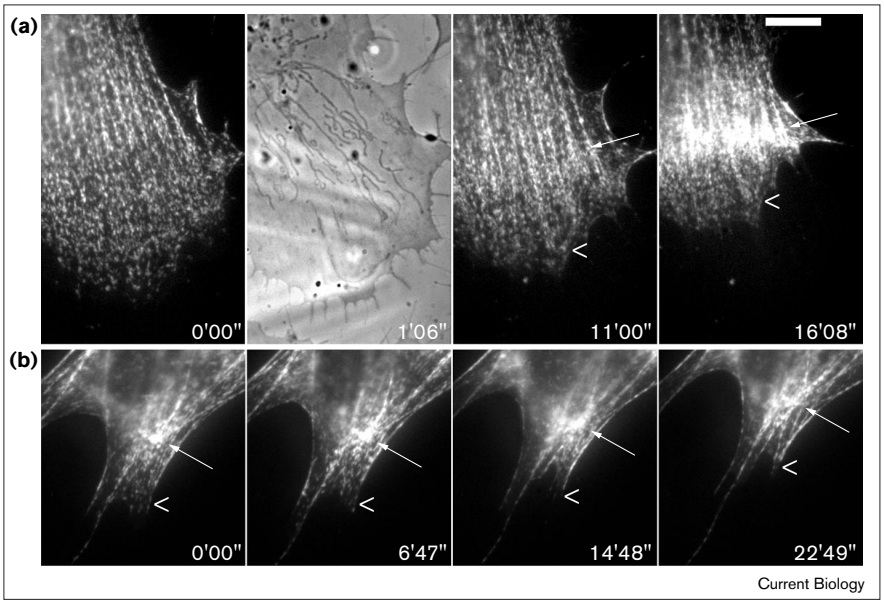
Enforced polarisation of a nocodazole-treated goldfish fibroblast on a flexible substrate. Fluorescence images show the movements of substrate-impregnated beads under a cell treated on one edge with ML-7 at the position indicated in the first frame (0 min). The cell edge, as seen in phase contrast (data not shown), is marked by a black line. The second and third frames show the boxed region from the first frame at higher magnification. The thin black line corresponds to the cell border at 0 min and the thick line to the border at 9 and 16 min. Bead displacements, compared to time 0, are indicated by the double-bead images connected by straight lines. Cell-edge retraction was associated with substrate relaxation (bead movement outwards). Scale bar in (a) represents 10 μm; in (b,c) 2 μm. See Supplementary material for movie.

the advance of a cell front in fibroblasts, driven by protruding lamellipodia, is not dependent on microtubules. Second, they support the idea that microtubules are required for the turnover of focal adhesions, as this was retarded during enforced movement in their absence.

Waterman-Storer *et al.* [2] showed that recovery of cells from nocodazole was associated with the activation of Rac. They speculated that Rac–GTP may be generated at the ends of microtubules, leading to the generation of membrane ruffling in the vicinity. Rather than being linked to microtubule ends, Rac activation under these conditions may be a result of the reciprocal balance between Rac and Rho activities [9–10], so that downregulation of the Rho pathway during microtubule recovery could lead to the activation of Rac and, in turn, to membrane ruffling. Alternatively, the upregulation of Rac during microtubule recovery might directly antagonise focal adhesion development by another route by causing the downregulation of myosin filament assembly via phosphorylation of the myosin heavy chain [11]. By this means, actin filament assembly into stress fibres could be suppressed and assembly into lamellipodia enhanced (see below). In this context, components of the Rac pathway represent potential candidates for signals delivered to and concentrated at adhesion sites by microtubules [4].

Protrusion of a cell in response to relaxation on the opposite side resembles the retraction-induced protrusion described previously for untreated fibroblasts [12,13]. In line with these findings, we suggest that trailing-edge release promotes the disassembly of the actin cytoskeleton

Figure 3



Differential shortening of stress fibre bundles during cell-edge retraction, monitored in goldfish fibroblasts injected with fluorescent smooth muscle myosin. **(a)** Nocodazole-treated fibroblast undergoing enforced cell-edge retraction in response to ML-7 applied to one edge at time 0. Phase-contrast image at time 1 min 6 sec shows the position of the micropipette; other frames show the distribution of myosin as fluorescence. Scale bar represents 10 μm. The change in the concentration of myosin shows that cell-edge retraction was associated with shortening of the proximal, but not the distal, regions of the stress-fibre bundles. **(b)** Spontaneous cell-edge retraction in a control fibroblast. Differential contraction occurs in regions of the stress fibres remote from the cell edge. See Supplementary material for movies.

for recycling of the component parts to the cell front. The simplest example of this type of recycling is provided by the keratocyte [14]. Cytoplasmic fragments released by this cell type can be induced to polarise and move in response to a mechanical stimulus. This leads to a self-sustaining segregation of two coupled regions — an actin-rich protruding front and a myosin-rich contracting rear. In this system, the adhesion sites formed are transient [15] and their dissociation is effected by the lateral contractile forces that build up at the rear [16] independently of microtubules [17]. Unlike keratocyte movement, the enforced movement of fibroblasts initiated by retraction of one edge is not self-sustaining. Continued challenges with inhibitor at the trailing tails were required to promote retraction. Additional regulatory mechanisms are thus required in fibroblasts to effect the release of trailing adhesions. These must be provided by microtubules. It is notable in this connection that the requirement for microtubules for polarisation and directional locomotion is specifically shown by those cell types that develop focal adhesions, with their associated actin stress-fibre bundles.

Consistent with the findings of Chrzanowska-Wodnicka and Burridge [18] we show that the relaxation of stress fibre assemblies in the region of adhesion sites results in adhesion release. We also show that the released bundles are still contractile and that their shortening drives retraction of the cell edge. In a recent study, the sliding of contact sites observed in some situations [19] was also correlated with shortening of the associated actin bundles. Our present observations indicate that relaxation at stress fibre termini could suffice to initiate release and disassembly of adhesion sites. In line with our earlier observations [4], we suppose that microtubules mediate this localised relaxation, and promote the turnover process by this route.

If microtubules promote release of adhesions at the rear, one might ask why they also target adhesions behind the protruding cell front [3]. Our present findings suggest that, by controlling stress fibre development at the front, microtubule-linked signals can locally influence the proportion of the free actin pool that is recycled into new stress fibres. If stress fibre development is inhibited or retarded, more of the local actin pool can be funnelled into the formation of lamellipodia [11]. In the case of forced migration without microtubules, the bulk supply of actin from the retracting rear is apparently sufficient to feed both lamellipodia formation and unleashed stress fibre assembly. Under normal conditions, the final polarised form of the cell will presumably depend on the sum of these regional modulations of adhesion-site dynamics, effected via the microtubule network.

Supplementary material

Supplementary material including figures illustrating concentration gradient of ML-7 during enforced motility; tubulin and actin distribution in the cell undergoing enforced motility; stress fibre dynamics in cells expressing α-actinin–EGFP; and the cell response to local application of GRGDS peptide, as well as movies corresponding to all figures and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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