http://www.jstage.jst.go.jp/browse/biophysics

BIOPHYSICS

Vol. 7, pp. 69–75 (2011)
doi: 10.2142/biophysics.7.69

Staurosporine induces lamellipodial widening in locomoting fish keratocytes by abolishing the gradient from radial extension of leading edge

Takafumi Mizuno[1] and Yuji Sekiguchi[1]

[1]*Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan*

Received 8 August, 2011; accepted 8 September, 2011

Fish epidermal keratocytes locomote along surfaces without overall cell size or shape changes, as kinematically described by the graded radial extension (GRE) model. We found that the cell size increased during locomotion after the addition of a low dose of staurosporine or K-252a, broad-spectrum protein-serine/threonine kinase inhibitors. Quantitative shape analysis showed that the cell size increase resulted from an increase in lamellipodial width, the maximal length perpendicular to the direction of the cell locomotion, whereas the lamellipodial length, along the locomotion direction, remained constant. Importantly, the gradient of radial extension in the leading edge disappeared during lamellipodial width increase. These results suggest that a special mechanism for producing graded radial extension of lamellipodia exists to keep cell size constant, and that a protein-serine/threonine kinase plays an important role in regulating this mechanism.

Key words: cell motility, cell shape, cell size, mathematical model, staurosporine

Amoeboid locomotion is one of the most basic functions of animal cells, and is involved in complex biological phenomena, including development, immune response, and cancer metastasis[1–3]. Although genetic engineering and advanced microscopy have provided us with a vast amount of knowledge about individual genes and the cellular localization of their products underlying motility machinery, we still do not understand the mechanism by which the molecules integrate themselves to produce whole-cell movement. One reason for this appears to be the difficulty of carrying out detailed analysis of their complex shape changes.

Fan-shaped keratocytes isolated from fish epidermis are especially well-studied locomoting cells in the context of biophysics, due to the simplicity and regularity of their mesenchymal mode locomotion on surfaces on which they do not change their overall shape or size. Their locomotion has been described mathematically based on the graded radial extension (GRE) model[4]. The principal concept of this model is that radial extension of the anterior half of the cell and radial retraction of the posterior half occur simultaneously in a graded manner, and perpendicular to the cell margins. This enables the cells to move without changing their shape or size.

Previously, we showed the GRE model was also applicable to the locomotion of pseudopod fragments of human polymorphonuclear leukocytes (PMNs)[5] and to that of *amiB*-null mutant cells of the cellular slime mold *Dictyostelium discoideum*[6]. While the original cells (PMNs and *D. discoideum*) typically show amoeboid mode locomotion with complex shape and size changes during their locomotion, the GRE model may describe the movements of individual parts of the cell and a keratocyte-like mutant. The GRE model may thus describe a universal principle underlying the mesenchymal mode of amoeboid cell locomotion.

However, the GRE model is merely a kinematic description of cell locomotion: the molecular mechanism behind the model, for example, the molecular machine for producing the gradient in leading edge extension or for synchronizing

Corresponding author: Takafumi Mizuno, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba 305-8566, Japan. e-mail: t.mizuno@aist.go.jp

the anterior extension and the posterior retraction remain unclear. Although one of the authors of the GRE model and coworkers have recently proposed a new model which can predict both cell shape and speed from calculations of actin network treadmilling in lamellipodia[7], the origin of the gradient remains unknown.

In this paper, we describe our recent finding that adding a low concentration of a broad-spectrum protein-serine/threonine kinase inhibitor (staurosporine or K-252a) causes an enlargement of the cell size. Further analysis showed that these inhibitors appear to abolish the gradient in radial extension of the leading edge of keratocyte lamellipodia. This suggests that a protein-serine/threonine kinase is involved in the gradient-forming mechanism of the radial extension of the leading edge, and that the gradient has a key role in keeping the cell size constant.

Materials and methods

Preparation of cells

Primary cultures of fish epidermal keratocytes were prepared as described previously[8]. Briefly, a goldfish *Carrasius auratus* was anesthetized with tricaine. A maximum of ten scales were removed from the fish and washed in RPMI1640 medium supplemented with 10% fetal calf serum and Antibiotic Antimycotic Solution (Sigma-Aldrich Co., St. Louis, MO, USA). Scales in 40 μl of the culture medium were sandwiched between two glass coverslips 25 mm in diameter and left standing for two hours in 0.5% CO_2 at room temperature. Next, the coverslips with scales were transferred into 2 ml of culture medium and left overnight in 0.5% CO_2. The coverslips, to which were attached many single moving keratocytes that had migrated away from the scales, were then washed with and kept in Fish Ringer's solution (112 mM NaCl, 2 mM KCl, 2.4 mM $CaCl_2$, 1 mM Tris buffer, pH 7.4) until use.

Protein-serine/threonine kinase inhibitors

Staurosporine, KT5720, and KT5823 were products of Aromone Labs (Jerusalem, Israel), and Ro 31-8220, SB-415286, KN-93, K-252a, and K-252b were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA), while ML-7 and Y-27632 were from EMD Biosciences, Inc (Merck KGaA, Darmstadt, Germany).

Optical microscopy

The coverslips to which cells attached were integrated in the observation chamber[9] on a Carl Zeiss Axiovert 35M inverted microscope (Carl Zeiss Inc., Jena, Germany). Phase contrast images were obtained by using a XC-999 CCD video camera and a Hamamatsu Argus-20 image processor (Hamamatsu, Japan), and recorded onto an analog videodisc by using a Pioneer VDR-V150 videodisc recorder (Tokyo, Japan). Video images were digitized and stored as TIFF-format files in a Macintosh computer (Apple Computer, Inc., Cupertino, CA, USA) equipped with a Scion LG-3 frame grabber card (Frederick, MD, USA), by using the public domain NIH Image 1.61 software (W. Rasband, available to Internet users from http://rsb.info.nih.gov/nih-image/).

Shape analysis of cells

The width and length of lamellipodia were measured manually by using the NIH Image software. Outlines of keratocytes were semi-automatically extracted from digitized phase-contrast images. The other motility parameters were calculated using the DIAS 2.0 (Soll Technologies, Iowa City, IA, USA) software. The "instantaneous velocity" of a keratocyte in the current frame n was calculated by drawing a vector from the centroid's position in the previous frame (n–1) and to the position in the next frame (n+1), and by dividing the vector length by 4 s (twice the time interval).

Results

Fragmentation of lamellipodia induced by staurosporine at higher concentrations

Verkhovsky *et al.* have reported the production of motile fragments of fish epidermal keratocyte pseudopods[10]. They found that treatment with staurosporine, a protein-serine/threonine kinase inhibitor (100 nM) or with KT5926, a myosin light chain kinase inhibitor (20 μM) induced elongation and fragmentation of lamellipodia of keratocytes from a fish, the black tetra, which was followed by formation of motile cytoplasts. The mechanism of cytoplast formation, however, remain unclear. We examined the effect of staurosporine at various concentrations on goldfish keratocytes.

A few minutes after the addition of staurosporine at higher concentrations (50–100 nM), the lamellipodia of goldfish keratocytes slightly elongated in the direction perpendicular to the direction of locomotion, and almost simultaneously cleaved into several fragments with stalks connecting them to the cell body (Fig. 1B), similar to those seen with black tetra keratocytes[10]. Freely-moving fragments, disconnected from the cell body, were also produced from goldfish keratocytes (Fig. 1C–E, Supplementary Movie 1), although these were rare (less than 1%). Like their parental keratocytes (Fig. 1A), the fragments showed persistent locomotion without changes in overall shape or size (Fig. 1C–E, Supplementary Movie 1).

Enlargement of lamellipodia induced by staurosporine at lower concentrations

On the other hand, treatment of goldfish keratocytes with lower concentrations (10–20 nM) of staurosporine did not induce any lamellipodial fragmentation or cytoplast formation. To our surprise, the treatment instead induced a dramatic increase in cell size in almost all the fan-shaped locomoting cells (Fig. 2A–D, Supplementary Movie 2, 3). Under our experimental condition, most keratocytes showed a fan-

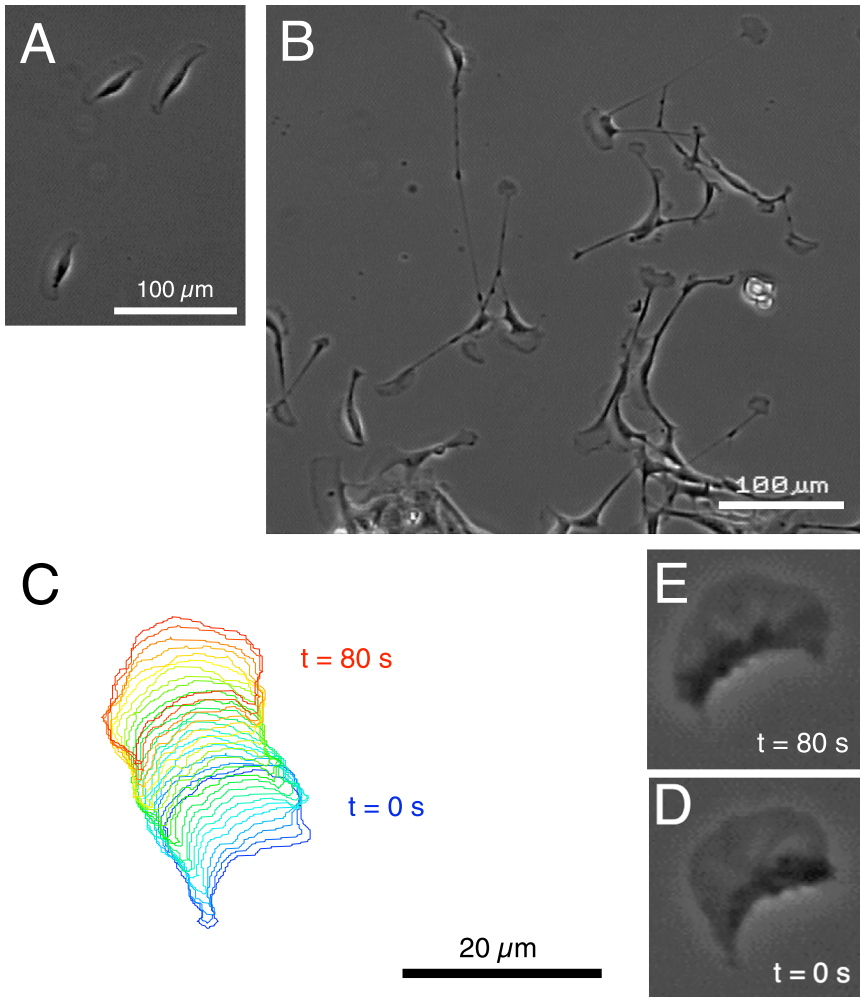

Figure 1 Fragmentation of lamellipodia induced by treatment with 100 nM staurosporine. Phase-contrast images of moving keratocytes before addition of staurosporine (A) and 60 minutes after addition (B). A stack of twenty-one successive outlines of a typical free-moving cytoplast caused by the inhibitor treatment (Supplementary Movie 1), separated by 4-s intervals with color from blue to red to denote time (0–80 s) (C). Phase-contrast images correspond to the first (D) and last (E) frames of the time-lapse sequence and are scaled to match to the outlines in C.

shaped morphology and high motility, whereas about 10% of keratocytes showed less-polarized fibroblast-like shapes and poor locomotion. Since the cell size increase on addition of staurosporine was not observed in these poorly motile cells, the enlargement of cells appeared to be related to cell locomotion.

Figure 2E shows changes in motility parameters of a typical cell treated with 10 nM staurosporine. The area of the cell increased by about 70% in 10 min during locomotion at a relatively constant instantaneous velocity about 10 μm/min. Quantitative analysis of cell shape changes revealed that the cell area increase was due to the elongation of the lamellipodial width and the end-to-end distance perpendicular to locomotion of the cell: the lamellipodial length, parallel to the locomotion, remained constant. It is noteworthy that the rate of lamellipodial width increase was not monotonic, and that there were two phases in the cell size changes: the "stationary phase", with cells locomoting while keeping their size constant, and the "widening phase", which showed an increase in lamellipodial width. These two phases alternated during cell locomotion.

The most remarkable characteristic of fan-shaped keratocytes is their persistent locomotion without changes in overall cell shape or size. These cells must thus have a special mechanism for maintaining their shape and size. The results described above suggest that staurosporine as a protein-serine/threonine kinase inhibitor may block one or more protein phosphorylation steps in the mechanism. We therefore tested several other inhibitors of protein-serine/threonine kinases, of which only staurosporine and K-252a, which have broad specificity, were found to be potent inducers of this phenomenon. However, K-252b (≤2.2 μM), another broad-spectrum inhibitor, was found not to induce similar effects. Treatment with more specific inhibitors including ML-7 (≤5 μM), Y-27632 (≤16.7 μM), KT5720 (≤5 μM), KT5823 (≤5 μM), Ro 31-8220 (≤100 nM), SB-415286

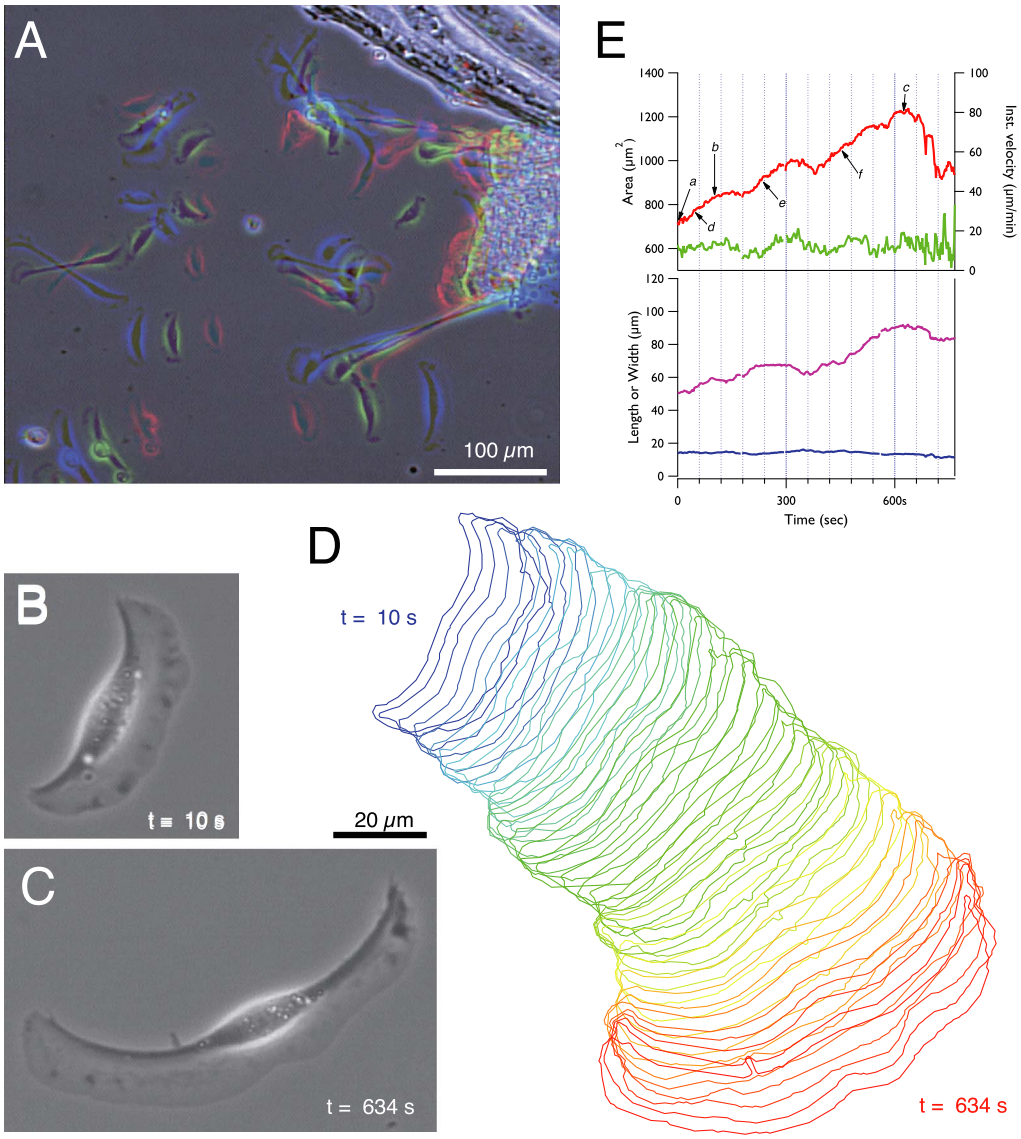

Figure 2 Lamellipodial enlargement induced by treatment with 10 nM staurosporine. (A) A merged phase-contrast image from three consecutive images of Supplementary Movie 2, separated by 200-s intervals (in the order of red, green and blue). (B) A phase-contrast image of a typical cell (Supplementary Movie 3) 10 s after addition of staurosporine. (C) An image of the same cell 634 s after addition. (D) Sequential cell outlines separated by 16-s intervals, colored from blue to red to represent time (10–634 s) after addition of staurosporine. Phase-contrast images B and C are scaled to match to the outlines in D. (E) Changes in area (plotted in red), instantaneous velocity (green), maximal width (magenta) and mean length (blue) of the lamellipod of the same cell as illustrated in panels B, C and D. Time points, a–f on the area plot are correspond to those in the panels in Figure 3.

(≤1 μM), and KN-93 (=2 μM) similarly did not induce lamellipodial enlargement or fragmentation (data not shown).

Lamellipodial morphology in stationary- and widening-phase cells

To learn how lamellipodial enlargement occurs, lamellipodial morphologies in the two phases, "stationary" and "widening," of the keratocytes treated with 10 nM staurosporine were compared in detail (Fig. 3). The lamellipodia in the stationary phase, locomoting with constant cell size, adopted a crescent shape typical of ordinary fan-shaped keratocytes, although they were more elongated than those of normal cells (Fig. 3A–C). Interestingly, the cells in the widening phase (Fig. 3D–F) were found to have a characteristic shape: that is, both sides of a widening lamellipod were nearly straight and perpendicular to the long arc of the leading front. "Square corners" were thus formed at both ends of the leading edge (Fig. 3D–F, arrow heads). The resulting morphology of the whole lamellipod thus resembled a part of a flat ring or a slice of Baumkuchen.

The GRE model is also applicable to staurosporine-treated cells in the stationary phase. As shown in Figure 3A–C, the graded speeds of the edge advancing along the leading edge, especially near both ends, were clearly detected

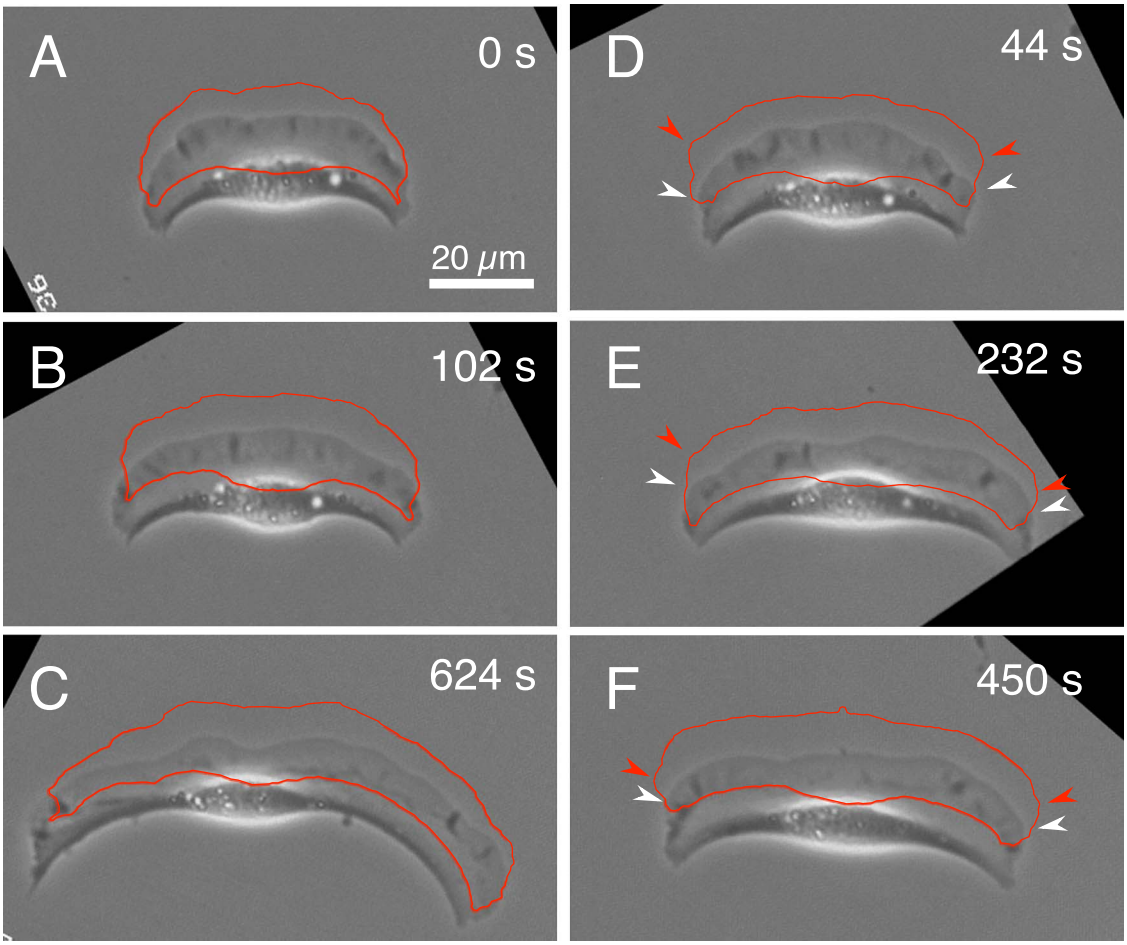

Figure 3 Lamellipodial morphology of a typical staurosporine-treated cell in distinct phases. Data for the same cell as in Fig. 2B–E and Supplementary Movie 3 are shown. Phase-contrast images of the cell and its outline after 32 s (red) are superimposed. (A–C) Stationary phases. Extension speeds at the leading edge showed a gradient along the leading edge. (D–E) Widening phases. Extension speeds were almost constant along the leading edge. Note that "square" corners were formed at each end of the half lamellipod without graded speed (arrow heads).

in the overlapping pictures of a phase contrast cell image and subsequently its outline (red) separated 32-s intervals: whereas the gradient in extension and retraction were completely abolished in the widening phase. The speeds of advancing edge were constant along the whole width of the lamellipodia (Fig. 3D–F). Thus, staurosporine appears to induce lamellipodial enlargement by abolishing the gradient in radial extension speeds of locomoting keratocytes.

Detailed observation of the transition processes between the stationary and widening phases revealed that morphological changes in lamellipodial ends occurred by extension speed changes along leading edges with no retraction of leading edges detected (Fig. 4).

Discussion

In this study, we found that treatment of goldfish keratocytes with low concentrations (10–20 nM) of staurosporine or K-252a induced a departure (lamellipodial widening) from the GRE model (Fig. 2, Supplementary Movie 2, 3). During this process, both ends of the lamellipodia changed from the ordinary curved forms into unusual rectilinear ones, and the whole lamellipodial morphology changed from the ordinary crescent shape into one shaped like a cut piece of Baumkuchen (Fig. 3).

As shown in Fig. 5, this phenomenon can be interpreted as the "gradient" of the radial extension of the lamellipod having been abolished in the GRE model. In fact, morphological analysis clearly showed the disappearance of the gradient from the edge extension speeds along leading edges during the widening phases (Fig. 3).

Because lamellipodia initially emerge from spherical or circular cells and then extend outward, their leading edges are always curved. The lamellipodial width must therefore have the fundamental property of widening during locomotion by radial extension perpendicular to the edge, and the morphology of the whole lamellipod must have a shape like a piece of Baumkuchen (Fig. 5B). Addition of an adequate gradient to the rate of radial extension and retraction in the lamellipodia converts their whole morphology into a cres-

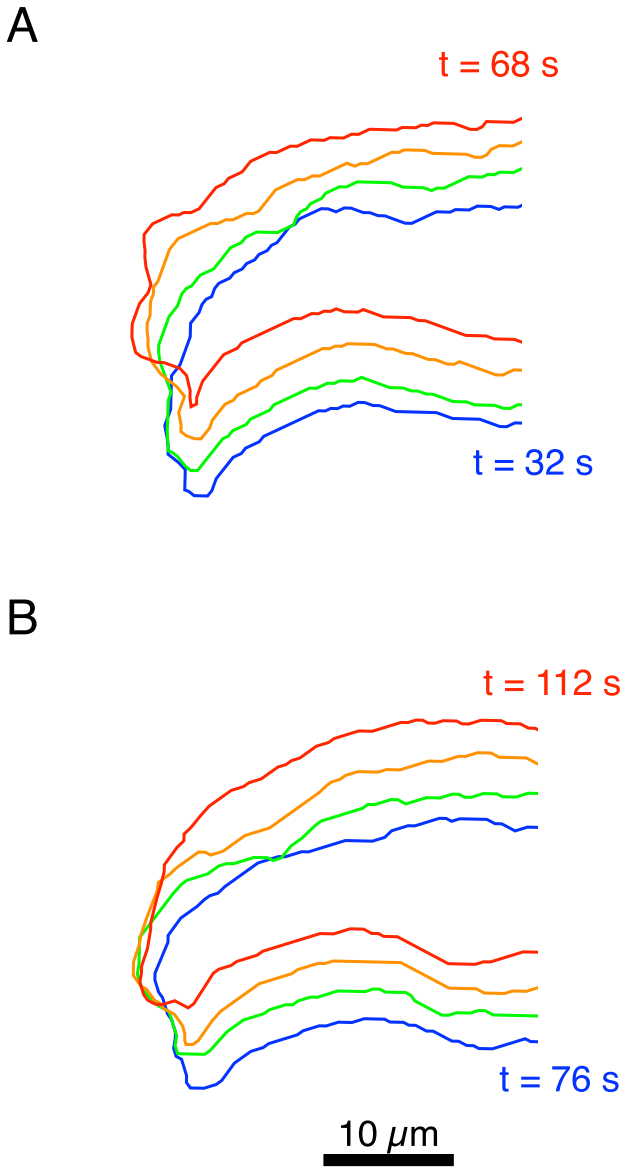

Figure 4 Transition between stationary and widening phases. Four successive outlines of a half-lamellipod separated by 12-s intervals are superimposed and colored from blue to red to represent time. Data for the same cell in as Fig. 2B–E, Fig. 3 and Supplementary Movie 3 are shown. (A) From stationary to widening phase. (B) From widening to stationary phase (outlines are mirrored for to allow comparison with A). Note that morphological changes in lamellipodial ends occurred as a result of extension speed changes along leading edges.

cent shape, and provides them with the ability to maintain a constant shape and size during locomotion (Fig. 5A). Thus, our result provides additional supporting evidence of that keratocyte locomotion is described in principle by the GRE model.

Keren *et al.*[7] have proposed a mechanism in which the graded density of local actin filaments along the leading edge produces the graded radial extension of the leading edge. However, they did not suggest how the density gradient of actin filaments emerges. Another possible candidate for the gradient-forming mechanism for lamellipodial extension may be a type of mechanical tension. It is known that the largest traction forces that fan-shaped keratocytes generate are located at both lateral ends of the lamellipod, oriented inward and perpendicular to the locomotion direction[11–15]. These strong inward forces may restrain and balance the outward forces from the widening actin meshwork at the lateral edges of a lamellipod.

Many examples of cell shape regulation by protein-serine/threonine kinases have been reported[16–18]. We have tested three broad-spectrum inhibitors of protein-serine/threonine kinases. Two of them, staurosporine and K-252a, induced lamellipodial widening in a similar manner. We thus believe that staurosporine causes lamellipodial widening by stimulation or inhibition of certain protein-serine/threonine kinases.

We also tested more specific kinase inhibitors. Those of protein kinases A, C, G, myosin light chain kinase, Rho-associated kinase, Ca^{2+}/calmodulin-dependent kinase, and glycogen synthase kinase-3 failed to induce lamellipodial widening. The true target of staurosporine may be a protein kinase different from those above. The inhibition of a specific combination of multiple kinases may be necessary to cause lamellipodial widening.

In the case of treatment with staurosporine at higher concentrations (50–100 nM), the large lamellipodia were fragmented into small pieces just after the beginning of widening, and motile cytoplasts were occasionally produced (Fig. 1). The effect was essentially the same as that seen in keratocytes from black tetra[10]. In spite of the presence of 100 nM staurosporine, the cytoplast moved straight without shape or size changes (Fig. 1C–E, Supplementary Movie 1). Lamellipodial widening occurred only in the presence of staurosporine at low concentrations.

Staurosporine at higher concentrations may disrupt other machinery that maintains the integrity of lamellipodia at a certain size or larger. Lamellipodial fragmentation may thus occur as soon as widening begins. Since the maximal width of these cytoplasts were about 10–20 μm, and they were much smaller than those of parental keratocytes, the residual surface tension of the cell membrane or cytoskeletal integrity might be sufficient to balance the widening force of the polymerizing actin meshwork. An additional machinery for counter-balancing the widening force may be necessary for larger cells.

Finally, our results suggest that the GRE model is more than a mathematical description of cell locomotion without shape and size changes, and that real cells are actually equipped with special machinery for generating a gradient for lamellipodial extension. Furthermore, it appears that a protein-serine/threonine kinase is involved in this. Elucidation of this protein kinase is of great importance for understanding the regulatory mechanism of the GRE model.

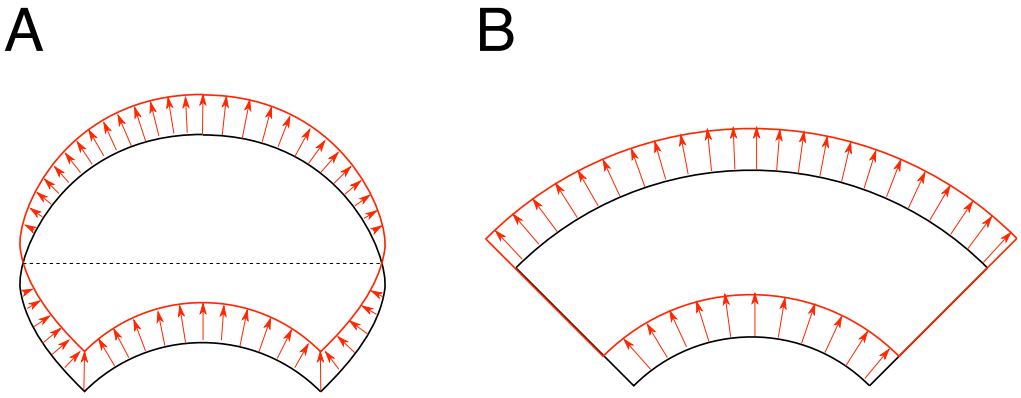

Figure 5 Models for illustrating keratocyte locomotion. (A) GRE model. Since leading edge extension and cell rear retraction occur perpendicularly to the cell margin at graded speeds (red arrows), cell sizes and shapes are maintained at their subsequent position (red margin). (B) Without a gradient in the extension speeds of the leading edge, cell sizes increase during locomotion.

Acknowledgements

We would like thank to Ms. T. Seki and Ms. Y. Sun of our institute for technical assistance, and Dr. T. Shimizu, Dr. Y. Asano, Dr. Y. Hiratsuka, Dr. A. Nagasaki, Dr. T. Q. P. Uyeda, Dr. K. Katoh, Ms. K. Yoshioka, and Dr. K. Kawasaki for their helpful discussions.

References

1. Pollard, T. D. & Cooper, J. A. Actin, a central player in cell shape and movement. *Science* **326**, 1208–1212 (2009).
2. Fletcher, D. A. & Mullins, R. D. Cell mechanics and the cytoskeleton. *Nature* **463**, 485–492 (2010).
3. Ridley, A. J. Life at the leading edge. *Cell* **145**, 1012–1022 (2011).
4. Lee, J., Ishihara, A., Theriot, J. A. & Jacobson, K. A. Principles of locomotion for simple-shaped cells. *Nature* **362**, 167–171 (1993).
5. Mizuno, T., Kagami, O., Sakai, T. & Kawasaki, K. Locomotion of neutrophil fragments occurs by graded radial extension. *Cell Motil. Cytoskeleton* **35**, 289–297 (1996).
6. Asano, Y., Mizuno, T., Kon, T., Nagasaki, A., Sutoh, K. & Uyeda, T. Q. P. Keratocyte-like locomotion in *amiB*-null *Dictyostelium* cells. *Cell Motil. Cytoskeleton* **59**, 17–27 (2004).
7. Keren, K., Pincus, Z., Allen, G. M., Barnhart, E. L., Marriot, G., Mogilner, A. & Theriot, J. A. Mechanism of shape determination in motile cells. *Nature* **453**, 475–480 (2008).
8. Mizuno, T., Sakai, T., Yoshioka, K. & Kawasaki, K. Difference-from-prediction imaging for high resolution shape analysis of locomoting cells. *Bioimages* **11**, 61–66 (2003).
9. Mizuno, T., Kawasaki, K. & Miyamoto, H. Construction of a thermotaxis chamber providing spatial or temporal thermal gradients monitored by an infrared video camera system. *Anal. Biochem.* **207**, 208–213 (1992).
10. Verkhovsky, A. B., Svitkina, T. M. & Borisy, G. G. Self-polarization and directional motility of cytoplasm. *Curr. Biol.* **9**, 11–20 (1999).
11. Lee, J., Leonard, M., Oliver, T., Ishihara, A. & Jacobson, K. A. Traction forces generated by locomoting keratocytes. *J. Cell Biol.* **127**, 1957–1964 (1994).
12. Burton, K., Park, J. H. & Taylor, D. L. Keratocytes generate traction forces in two phases. *Mol. Biol. Cell* **10**, 3745–3769 (1999).
13. Oliver, T., Dembo, M. & Jacobson, K. A. Separation of propulsive and adhesive traction stresses in locomoting keratocytes. *J. Cell Biol.* **145**, 589–604 (1999).
14. Doyle, A. D., Marganski, W. & Lee, J. Calcium transients induce spatially coordinated increases in traction force during the movement of fish keratocytes. *J. Cell Sci.* **117**, 2203–2214 (2004).
15. Fournier, M. F., Sauser, R., Ambrosi, D., Meister, J.-J. & Verkhovsky, A. B. Force transmission in migrating cells. *J. Cell Biol.* **188**, 287–297 (2010).
16. Sells, M. A., Boyd, J. T. & Chernoff, J. p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145**, 837–849 (1999).
17. Koivisto, L., Alavian, K., Häkkinen, L., Pelech, S., McCulloch, C. A. & Larjava, H. Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes. *J. Cell Sci.* **116**, 3749–3760 (2003).
18. Woodrow, M. A., Woods, D., Cherwinski, H. M., Stokoe, D. & McMahon, M. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf → MEK → ERK pathway. *Exp. Cell Res.* **287**, 325–338 (2003).