Tension-Dependent Formation of Stress Fibers in Fibroblasts: A Study Using Semi-Intact Cells*

Hiroaki HIRATA**, Hitoshi TATSUMI***,**** and Masahiro SOKABE**,***,†

Stress fiber is a bundle of actin filaments, which also contains myosin and cross-linking proteins. It remains unclear how stress fibers are formed from their main components, actin and myosin. To address this question we examined the process of formation of stress fibers in a model system, a semi-intact cell. Fibroblasts were treated with a Rho kinase inhibitor to disorganize stress fibers. These cells were permeabilized and used as semi-intact cells in this study. When these cells were treated with ATP and Ca^{2+}, stress fibers were restored within 15 min. Motion analysis of actin filaments labeled with quantum dots during formation of stress fibers revealed actin filaments were gradually assembled while they were moving toward the center of the cell. This suggests that ATP-driven tension influences the stress fiber formation. The ATP-driven tension was mimicked in this study by artificial centripetal traction force, which was carried out by dragging the micropipette attached on the cell surface. Stress fibers were formed by the traction force application. These results suggest that tension in actomyosin meshwork is an important element in stress fiber formation.

Key Words: Biomechanics, Image Processing, Optical Measurement, Stress Fiber, Semi-Intact Cell, Traction Force, Molecular Machine, Self-Organization

1. Introduction

In fibroblasts or endotherial cells, bundles of actin filaments, called stress fibers, are developed. Stress fiber is a long linear structure ranging over almost entire cell length and is highly oriented along the cell axis. The ends of stress fibers are anchored to cell-to-substrate contact regions, focal adhesions. Stress fiber is contractile[1),(2)] and it exerts traction force on focal adhesions[3)]. This force exertion is crucial for regulating cell adhesion[4)] and remodeling extracellular matrix[5)]. Dynamic nature of stress fibers is responsible for cell motility and remodeling of cell shape[6)-(8)].

Formation of stress fibers is stimulated by some extracellular factors, such as lysophosphatidic acid[9)] and thrombin[10)]. These factors activate a small GTPase, Rho, and its effecter proteins. Rho kinase/ROCK is a major Rho effecter protein and is responsible for stress fiber formation[11),(12)]. Rho kinase enhances phosphorylation of myosin light chain[13),(14)], resulting in activation of actomyosin and force generation. Another major Rho effecter, mDia, cooperates with Rho kinase in stress fiber formation, probably through the regulation of actin polymerization[15)].

Although a part of signaling cascade for regulation of stress fiber formation has been revealed, it remains largely unclear how stress fibers are constructed from their components including actin and myosin. Since globular actin (G-actin) and filamentous actin (F-actin) coexist in a cell, G-actin or F-actin may contribute to stress fiber formation. Some studies suggested contribution of localized de novo actin polymerization to stress fiber formation; when fluorescently labeled G-actin is introduced into living cells or permeabilized cells, the labeled actin is assembled primarily at the ends of stress fibers during stress fiber development[6),(16),(17)]. On the other hand, Machesky and Hall[18)] showed delayed incorporation of Cy3-labeled actin into

* Received 24th May, 2004 (No. 04-4126)
** Department of Molecular Physiology, National Institute for Physiological Science, Okazaki, Aichi 444–8585, Japan
*** Department of Physiology, Nagoya University Graduate School of Medicine, 65 Tsurumai Showa-ku, Nagoya, Aichi 466–8550, Japan
**** CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332–0012 Japan
† ICORP, Cell Mechanosensing Project, Japan Science and Technology Agency, 65 Tsurumai Showa-ku, Nagoya, Aichi 466–8550, Japan.
E-mail: msokabe@med.nagoya-u.ac.jp

stress fibers during stress fiber formation, concluding that stress fibers are assembled primarily by bundling of pre-existing F-actin. However, as pointed out by Watanabe et al.[15], contribution of F-actin could not be distinguished completely from that of G-actin in Machesky and Hall's study. The actual contribution of G-actin and F-actin to stress fiber formation is not evaluated yet, since complete separation of actin polymerization from F-actin reorganization is practically difficult in living cells.

It has been postulated that formation of stress fibers depends on mechanical tension. Circumstantial evidence supports this hypothesis as follows; activation of the actomyosin interaction, which generates contractile force, was reported to be necessary for stress fiber formation[19]. Fibroblasts within anchored collagen gel develop stress fibers but not within floating gel[20], that is probably because the floating gel is insufficient to sustain mechanical tension. However, it is not resolved yet how mechanical forces work on components of stress fibers in constructing stress fibers.

In this study we examined the process of stress fiber formation. To overcome the technical difficulties to manipulate the molecules responsible for stress fiber formation in living cells, we used semi-intact cells, where majority of soluble cellular components, including actin monomers and ATP, are excluded while actin filaments and their associating proteins remain in the cell[21]. Moreover, semi-intact cells enabled us to introduce molecules, which are permeable through the semi-intact cell membrane, into the intracellular space. Here we report that bundles of actin filaments are reconstructed from pre-existing actin filaments in semi-intact fibroblasts. During the bundle formation actin filaments were gradually assembled as moving toward the center of the cell. In addition, the artificially applied centripetal traction force could also arrange pre-existing actin filaments into stress fibers, suggesting that tension in F-actin meshwork is an essential factor in stress fiber formation.

2. Materials and Methods

2.1 Materials

Y-27632, a specific Rho kinnase inhibitor[22], was purchased from Calbiochem (San Diego, CA). Anti-non-muscle myosin monoclonal antibody was from Abcam Ltd. (Cambridge, UK). Anti-α-actinin monoclonal antibody was from ICN Pharmaceuticals, Inc. (Costa Mesa CA). Anti-α-tubulin monoclonal antibody was from Cedarlane Laboratories Ltd. (Hornby, Canada). Streptavidin-conjugated quantum dot (Qdot) was from Quantum Dot Co. (Hayward, CA). Alexa488- and biotinXX-conjugated phalloidin and Alexa546-conjugated anti-mouse IgG polyclonal antibody were from Molecular Probes, Inc. (Eugene, OR).

2.2 Cell culture

The human foreskin fibroblast cell line Hs 68 was cultured in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Nipro, Osaka, Japan) at 37°C in 5% CO_2. For experiments cells were grown on the glass coverslip.

2.3 Preparation of semi-intact cells

Cells grown for 1 day were treated with 20 μM Y-27632 in DMEM/FBS for 1 h at 37°C to disorganize pre-existing stress fibers. To prepare semi-intact cells with stable and visible actin filaments, Y-27632-treated cells were permeabilized with 0.003% digitonin in the presence of 3.3 U/mL Alexa488-phalloidin in working solution (125 mM potassium acetate, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$, 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 12 mM glucose, 0.1 μM phenylarsine oxide, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL chymostatin, 10 mM dithiothreitol; pH = 7.0) for 1 min at room temperature. To prepare semi-intact cells with actin filaments labeled with Qdots, Y-27632-treated cells were permeabilized for 1 min with 0.003% digitonin in the presence of 0.33 μM biotinXX-phalloidin, followed by a treatment with 0.1 nM streptavidin-conjugated Qdot for 1 min. Qdots were treated in the presence of 1% BSA. After washing in working solution the semi-intact cells were used for an experiment. To activate the actomyosin interaction, 0.1 mM ATP and 0.1 mM $CaCl_2$ were applied to the semi-intact cells.

2.4 Single Qdot tracking experiment

Time-sequence images of Qdot were acquired at time interval as indicated in individual figure legends. Position of individual Qdots was determined frame-by-frame by calculating the centroid of the fluorescence image of Qdot. Tracking duration of individual Qdot motion was indicated in individual figure legends. For calculation of an area of distribution of each Qdot, the two-dimensional mean square displacement, $\langle r^2 \rangle$, was calculated for each Qdot, where r denotes the displacement of the bead during each time interval, t, and $\langle\ \rangle$ denotes the average over the tracking duration. In the case that distribution of Qdot was confined, the $\langle r^2 \rangle - t$ curve exhibited convex curvature and reached a plateau value (here referred as $L^2/6$[23]). The L^2 value was determined from the $\langle r^2 \rangle$ values averaged over $t = 4 - 60$ s and referred as the index of the area of distribution of each Qdot.

2.5 Cell-dragging experiment

Glass capillaries with diameter of 1 mm (G-1, Narishige Co., Tokyo) were pulled with a Flaming/Brown micropipette puller (P-97, Sutter Instrument Co., Novato, CA). The micropipettes were bent in middle by burning them with a gas burner. The micropipettes were treated with 3-aminopropyltriethoxysilane, dried, coated

with 20% glutaraldehyde for 5 min at room temperature and washed before use. In the presence of 0.1 mM ATPγS and 0.1 mM $CaCl_2$ the tip of the micropipette was horizontally placed on the boundary region between the lamella and the cell body of the semi-intact cell, and then it was dragged toward the center of the cell by using a micromanipulator (MC-35A, Narishige Co.). This enabled us to apply centripetal traction force on the semi-intact cell.

2.6 Immunostaining

For immunostaining, intact and semi-intact cells were fixed with 3.7% formaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 5 min, followed by blocking with 1% BSA and 1% glycine for 30 min and were subjected to immunostaining. Treatment with antibodies were for 40 min. Dilutions were 1 : 100 for primary antibodies and 1 : 50 for secondary antibody with working solution including 1% BSA. Actin filaments of intact cells were stained with 0.67 U/mL Alexa488-phalloidin for 40 min.

2.7 Fluorescence microscopy

Observations were performed with an IX70 epi-fluorescence microscope (OLYMPUS, Tokyo) equipped with an oil immersion objective (NA 1.40, 100×; PlanApo, OLYMPUS) and a charge-coupled device camera (Micromax, Princeton Instruments, Trenton, NJ). The fluorescent probes were illuminated with a xenon lamp, and Qdots with a solid state blue laser (λ = 473 nm; HK-5512-01, SHIMADZU Co., Kyoto, Japan). Images were acquired and analyzed with the software MetaMorph (Universal Imaging Co., Downingtown, PA).

3. Results

Human foreskin fibroblasts grown for 1 day exhibited well-developed stress fibers (Fig. 1 (a)). After a treatment with 20 μM Rho kinase inhibitor, Y-27632, for 1 h at 37°C, most of stress fibers were lost, and large lamellae were protruded (Fig. 1 (b), arrows). Stress fibers recovered after removal of Y-27632 and following incubation for 2 h at 37°C (Fig. 1 (c)), showing disorganization of stress fibers by Y-27632-treatment was reversible. In lamellae developed after the treatment with Y-27632, there were no apparent actin filament bundles but blur F-actin was observed ((Fig. 1 (d)). Only 15 min after the removal of the inhibitor, stress fibers were formed in the lamella (Fig. 1 (e)). However, in cell bodies, stress fibers were not formed clearly (data not shown).

To examine the molecular mechanism of formation of stress fibers, we analyzed the stress fiber formation in a semi-intact cell system, in which we can manipulate molecular composition inside the cell. The effect of free actin monomers can be excluded in the semi-intact cell experiments since they presumably diffuse away through the permeabilized plasma membrane. Cells were treated with Y-27632 for 1 h to disorganize stress fibers and then permeabilized with 0.003% digitonin in the presence of

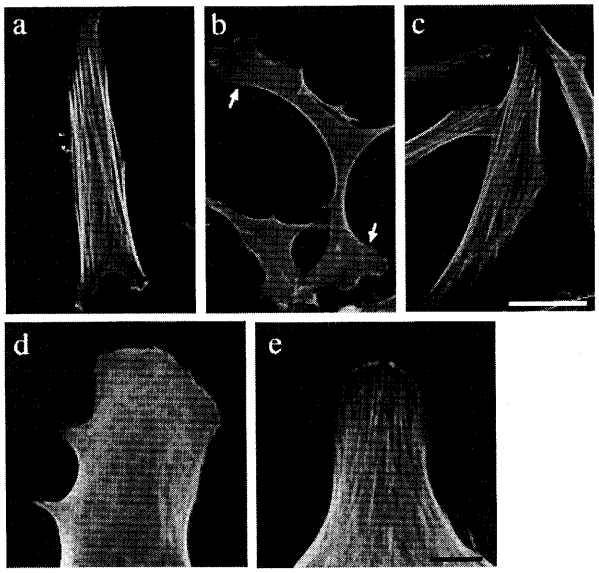

Fig. 1 Reversible disorganization of stress fibers by a Rho kinase inhibitor in intact cells. (a): F-actin in a human foreskin fibroblast stained with Alexa488-phalloidin. (b): F-actin in a cell after a treatment with 20 μM Y-27632, a specific Rho kinase inhibitor, for 1 h at 37°C. (c): F-actin in a cell treated with 20 μM Y-27632 for 1 h, followed by washing out Y-27632 and incubation for 2 h at 37°C. (d): F-actin in a lamella of a cell treated with 20 μM Y-27632 for 1 h. (e): F-actin in a lamella of a cell treated with 20 μM Y-27632 for 1 h, followed by washing out Y-27632 and incubation for 15 min at 37°C. Bars, 50 μm in (a–c), 10 μm in (d) and (e)

3.3 U/mL Alexa488-phalloidin to make semi-intact cells with fluorescently labeled F-actin. Figure 2 (a) shows a lamella of a semi-intact cell (arrow), in which blur F-actin staining was exhibited, as seen in fixed intact cells (Fig. 1 (d)). Such blur F-actin staining corresponds to F-actin meshwork. When 0.1 mM ATP and 0.1 mM $CaCl_2$ were applied to this semi-intact cell, bundles of actin filaments appeared in the lamella within 15 min (Fig. 2 (b), arrows). Time lapse imaging of such a process revealed that the pre-existing F-actin was gradually rearranged into bundles (Fig. 2 (c)). These results indicate that actin filament bundles, stress fibers, can be formed from pre-existing actin filaments without *de novo* actin polymerization.

Immunofluorescence staining revealed that the actin filament bundles formed in semi-intact cells contain myosin (Fig. 3 (a) and (b)) like as in intact cells (Fig. 3 (c) and (d)). In semi-intact cells α-actinin in F-actin bundles was scarce (data not shown) and microtubules were fragmented (Fig. 3 (e)), suggesting that α-actinin and microtubules are not essential for the formation of stress fibers in semi-intact cells.

To track the motion of actin filaments during stress fiber formation, actin filaments in semi-intact cells were sparsely labeled with Qdots. Qdot is a nano-crystal of semiconductor with bright fluorescence and high photo-

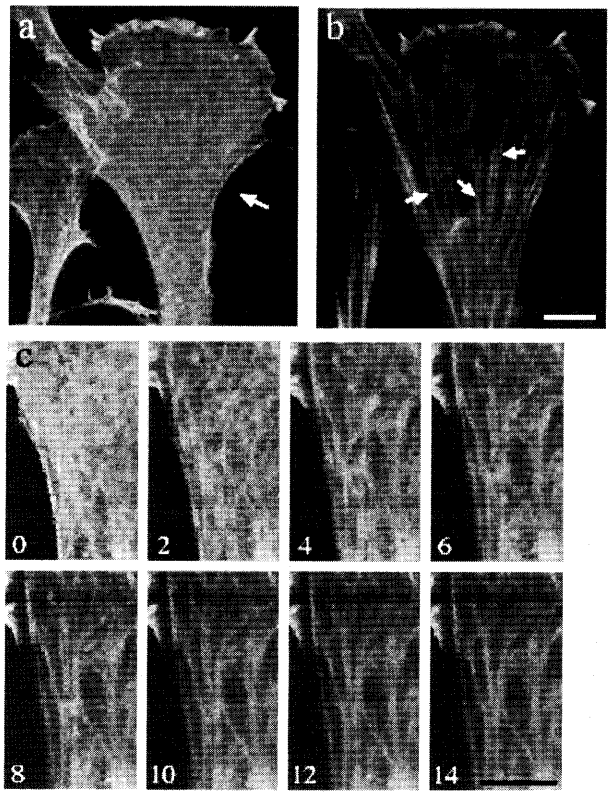

Fig. 2 Formation of actin filament bundles in semi-intact cells induced by ATP and Ca^{2+}. Human foreskin fibroblasts treated with 20 μM Y-27632 for 1 h were permeabilized with 0.003% digitonin in the presence of Alexa488-phalloidin. (a): F-actin in a lamella of a non-fixed semi-intact cell. (b): Same field as in (a) but 15 min after the application of 0.1 mM ATP and 0.1 mM $CaCl_2$. Actin filament bundles were formed (arrows). (c): Time lapse images of F-actin in a lamella of a non-fixed semi-intact cell after application of 0.1 mM ATP and 0.1 mM $CaCl_2$. Elapse time after the application of ATP and $CaCl_2$ is indicated by numbers in minutes. Bars, 10 μm

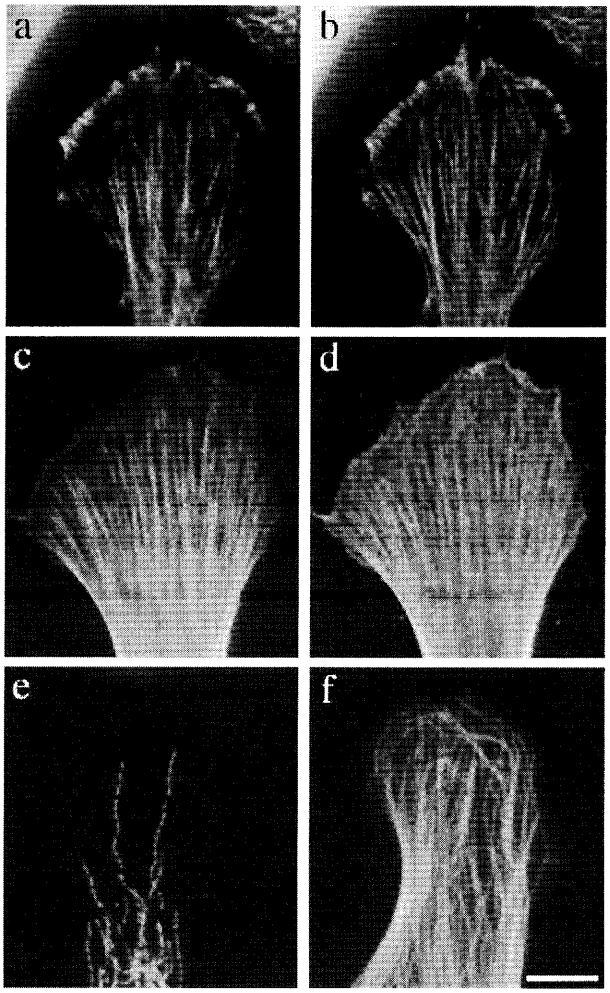

Fig. 3 Distribution of myosin and microtubules in lamellae of semi-intact cells and intact cells. Semi-intact cells were prepared as in Fig. 2. (a): F-actin in a lamella of a semi-intact cell treated with 0.1 mM ATP and 0.1 mM $CaCl_2$ for 15 min. (b): Myosin in the same field as in (a). (c): F-actin in a lamella of an intact cell treated with 20 μM Y-27632 for 1 h, followed by washing out Y-27632 and incubation for 15 min at 37°C. (d): Myosin in the same field as in (d). (e): α-tubulin in a lamella of a semi-intact cell. (f): α-tubulin in a lamella of an intact cell treated with 20 μM Y-27632 for 1 h. Bar, 10 μm

stability[24], therefore, it can be used as a fluorescent marker for long-term tracking. F-actin decorated with biotin-phalloidin could be labeled specifically with streptavidin-Qdots owing to the sepecific and tight interaction between streptavidin and biotin. Figure 4 (a) shows a lamella of a semi-intact cell treated with biotin-phalloidin and then with streptavidin-Qdots. When semi-intact cells were treated with phalloidin instead of biotin-phalloidin in control experiments, few streptavidin-Qdots were bound to the semi-intact cells (Fig. 4 (b)), indicating that labeling of actin filaments with Qdots was highly specific. Figure 4 (c) and (d) show the typical trajectories of the single Qdots fixed on the glass surface and those bound to F-actin in a lamella of a semi-intact cell in the absence of ATP and Ca^{2+}, respectively. To fix Qdots on the glass surface, Qdot suspension was dropped onto the glass and air-dried. The area of distribution ($L^2 = 0.006\,4 \pm 0.003\,8\ \mu m^2$, $n = 7$; see Materials and methods) of Qdots bound to F-actin in semi-intact cells in the absence of ATP and Ca^{2+} was not significantly different from that ($L^2 = 0.005\,3 \pm 0.002\,6\ \mu m^2$, $n = 6$) of Qdots fixed on the glass surface ($p > 0.55$), indicating that Qdots bound to F-actin in semi-intact cells were kept still in the absence of ATP and Ca^{2+}. After application of 0.1 mM ATP and 0.1 mM $CaCl_2$, Qdots bound to F-actin in lamellae of semi-intact cells began to move (a typical trajectory is shown in Fig. 4 (e)). Figure 4 (f) shows trajectories of neighboring 4 Qdots in a lamella after the application of ATP and Ca^{2+}; every Qdot moved from upper to lower, corresponding to the movement toward the center of the cell. Trajectories of 4 Qdots became closer each other as they moved toward the center of the

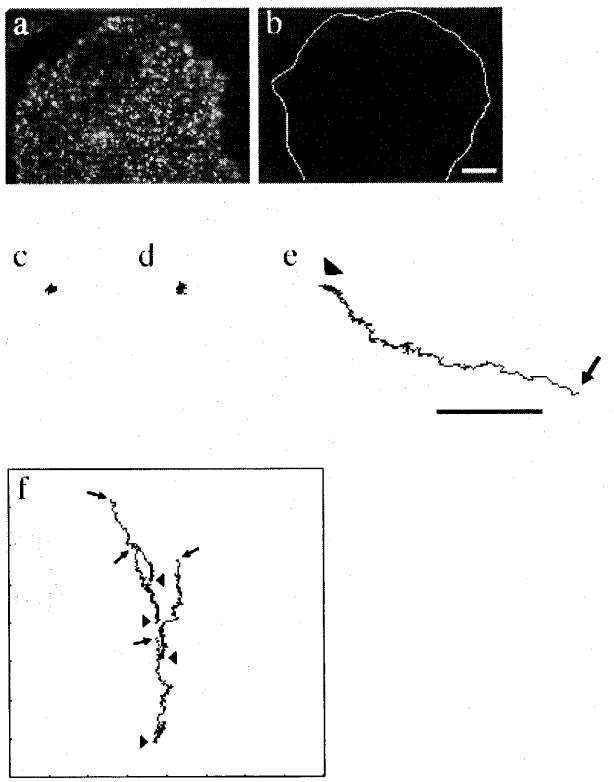

Fig. 4 Analysis of motion of Qdot bound to F-actin in a semi-intact cell during treatment with ATP and Ca^{2+}. Fibroblasts treated with 20 μM Y-27632 for 1 h were permeabilized with 0.003% digitonin in the presence of biotin-phalloidin. (a): A lamella of a streptavidin-Qdot-treated semi-intact cell, in which F-actin was decorated with biotin-phalloidin. (b): A lamella of a streptavidin-Qdot-treated semi-intact cell, in which F-actin was decorated with non-conjugated phalloidin instead of biotin-phalloidin. The line drawing represents the boundary of the semi-intact cell obtained from its differential interference contrast image. (c–e): Trajectory of Qdot fixed on the glass surface (c), trajectory of Qdot bound to F-actin in a lamella of a semi-intact cell in the absence of ATP and $CaCl_2$ (d) and trajectory of Qdot bound to F-actin in a lamella of a semi-intact cell in the presence 0.1 mM ATP and 0.1 mM $CaCl_2$ (e). Motion of Qdot was tracked in 2 s interval for 240 s in (c) and (d) or for 800 s in (e). In (e), motion of Qdot was tracked from immediately after the application of ATP and Ca^{2+}, and Qdot moved from the lower right to the upper left. An arrow and an arrowhead indicate start and end points of the trajectory of Qdot, respectively. (f): Trajectories of 4 Qdots bound to F-actin in a lamella of a semi-intact cell in the presence of 0.1 mM ATP and 0.1 mM $CaCl_2$. Motion of Qdots was tracked from immediately after the application of ATP and Ca^{2+} in 2 s interval for 800 s. Every Qdot moved from upper to lower, corresponding to the movement toward the center of the cell. Arrows and arrowheads indicate start and end points of the trajectories of Qdots, respectively. Axis ticked every 1 μm. Bars, 10 μm in (a) and (b), 1 μm in (c–e)

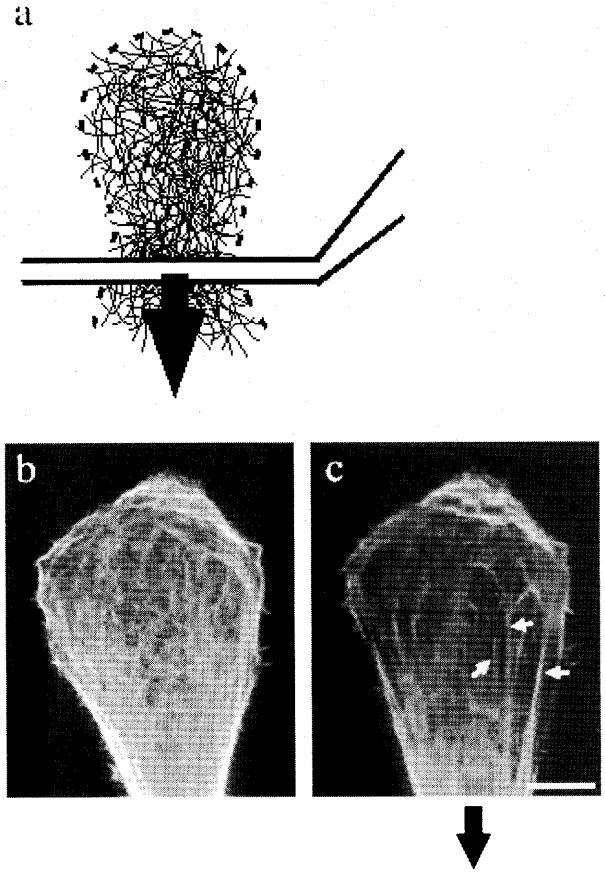

Fig. 5 Formation of stress fibers in a semi-intact cell induced by artificially applied mechanical force. Fibroblasts treated with 20 μM Y-27632 for 1 h were permeabilized with 0.003% digitonin in the presence of Alexa488-phalloidin. (a): Scheme depicting the method for force application. A bent glass micropipette, which was coated with glutaraldehyde, was horizontally placed on the boundary between the lamella and the cell body of a semi-intact cell, and then it was dragged toward the center of the cell. (b) and (c): F-actin in a lamella of a non-fixed semi-intact cell before (b) and after (c) dragging the micropipette. The micropipette is out of these images. After the application of artificial force stress fibers appeared (white small arrows). A large black arrow indicates the direction of dragging the micropipette. Bar, 10 μm

cell, suggesting actin filaments were bundled gradually. The directed movement of Qdots suggests a certain force that pulled the F-actin meshwork in a lamella toward the center of the cell and may facilitate the bundling of actin filaments.

To directly examine the role of traction force in the formation of actin filament bundles, we artificially pulled the F-actin meshwork toward the center of the cell. A glass micropipette coated with glutaraldehyde was horizontally attached on the boundary between a lamella and a cell body of a semi-intact cell. Then the pipette was dragged toward the center of the cell to apply traction force onto

the F-actin meshwork, as shown in Fig. 5 (a). To release F-actin from the rigor state actomyosin interaction but prohibit actomyosin-dependent force generation, experiments were performed in the presence of ATPγS, a non-hydrolyzable analog of ATP[25]. Application of 0.1 mM ATPγS and 0.1 mM $CaCl_2$ alone did not cause formation of bundles of actin filaments; Fig. 5 (b) shows blur F-actin staining in a lamella before applying traction force. Figure 5 (c) shows the same lamella after an application of traction force, in which the pre-existing F-actin was rearranged and stress fibers were formed (arrows). This result indicates that centripetal traction force is essential for the formation of stress fibers from pre-existing F-actin meshwork in semi-intact cells. In the absence of ATPγS the stress fibers were hardly formed by the artificial traction force, suggesting that actin filaments are rigidly cross-linked by myosin in a rigor state and are not rearranged by the traction force.

4. Discussion

In this study we demonstrated that bundles of actin filaments were formed by application of ATP and Ca^{2+} and by artificial traction force in semi-intact cells. The actin filament bundles formed in semi-intact cells contained myosin as stress fibers in intact cells, suggesting that stress fibers are reconstructed in semi-intact cells. The semi-intact cell developed in this study is useful for studying on the mechanism of stress fiber formation.

Plasma membrane of the semi-intact cells permeabilized with digitonin was permeable for soluble proteins and streptavidin-conjugated Qdots (ca. 10 nm in diameter). When fluorescently labeled G-actin was applied to semi-intact cells and then washed out, the exogenous G-actin was incorporated into actin filaments without distribution all over the intracellular space (unpublished observation), suggesting that G-actin is not left inside semi-intact cells after washing it out. Therefore, it is conceivable that rearrangement of F-actin is responsible for the formation of stress fibers in semi-intact cells.

In the cytoskeletal system there exist microtuble-associated motor proteins, i.e. kinesins and dyneins, and actin filament-associated motor proteins, i.e. myosins[26]. Since microtubules in semi-intact cells were fragmented, it is unlikely that microtuble-associated motor proteins generate force to reorganize the F-actin meshwork in a large scale. Non-muscle myosin generates force in the presence of ATP and Ca^{2+}[27]. Actomyosin-dependent force generation seems to be responsible for reorganization of the pre-existing F-actin meshwork into stress fibers in semi-intact cells during treatment with ATP and Ca^{2+}.

In single Qdot tracking experiments, Qdots bound to F-actin in lamella region moved in centripetal direction during treatment with ATP and Ca^{2+}, however, those bound to F-actin in the cell body did not (unpublished observation), indicating that F-actin meshwork was pulled toward the cell body only in the lamella. Stress fibers were formed in the lamella but not in the cell body in a semi-intact cell. Same observation was made in intact cells 15 min after the recovery from the Rho kinase inhibitor. A lamella may be a region where the F-actin meshwork is subjected to mechanical traction force, and consequently, formation of stress fibers is enhanced.

The ATP-driven traction force was mimicked by artificially applied traction force onto the F-actin meshwork. The artificially applied traction force rearranges the F-actin meshwork into actin filament bundles oriented along the traction direction, which are the same as the stress fibers formed in semi-intact cells in the presence of ATP. This suggests that mechanical tension in F-actin meshwork is an important element in stress fiber formation.

When ATP and Ca^{2+} were applied, stress fibers were formed in lamellae of almost all semi-intact cells. In contrast, artificially applied traction force sometimes failed to form stress fibers. This suggests factor(s) other than traction force affects stress fiber formation during treatment with ATP and Ca^{2+}. Centripetal traction force may roughly reorganize the F-actin meshwork and concomitantly occurred local zippering of actin filaments by myosin molecules along actin filaments would facilitate stress fiber formation.

In intact cells, stress fiber formation is regulated by Rho kinase[11),(12)], which enhances phosphorylation of myosin light chain[13),(14)]. Constitutively active form of Rho kinase induces stress fiber formation without activation of Rho[11),(12)]. However, stress fibers induced by Rho kinase are locally accumulated, which are distinct from stress fibers induced by activated Rho[15),(28)]. Active mDia, another effecter of Rho, induces the complete phenotype of stress fibers in cooperation with Rho kinase[15)], suggesting that in addition to Rho kinase-dependent activation of actomyosin, mDia-dependent actin polymerization is necessary for completion of stress fiber formation. While stress fibers were reformed completely 2 h after removal of the Rho kinase inhibitor in intact cells, actin filament bundles were poor in the cell body of a semi-intact cell, in which actin polymerization was excluded, even 2 h after the application of ATP and Ca^{2+} (unpublished observation). This incompleteness of formation of stress fibers in the cell body may be due to the lack of mDia-dependent actin polymerization in semi-intact cells. The stress fibers formed in lamellae may work as precursors of mature stress fibers extending over almost entire cell length and mDia-induced supply of additional actin filaments may thicken and elongate the stress fibers.

In intact cells, stress fiber formation is coupled to formation of focal adhesions. It was revealed by reflection interference microscopy that stress fiber formation was not accompanied by formation of focal adhesions in

semi-intact cells (unpublished observation). Riveline et al. showed that mDia and actin polymerization are required for formation of focal adhesions[29]. Introduction of mDia-dependent actin polymerization into semi-intact cells would be useful to acquire information about formation of focal adhesions accompanied by stress fiber formation.

Acknowledgement

We thank Dr. K. Hayakawa for helpful advice. This work was supported by grants for Scientific Research (13480216), Scientific Research on Priority Areas (15086270), and Creative Scientific Research (16GS0308) from MEXT, and a grant from Japan Space Forum to MS, and Scientific Research (14580769) from MEXT to HT.

References

(1) Isenberg, G., Rathke, P.C., Hülsmann, N., Franke, W.W. and Wohlfarth-Bottermann, K.E., Cytoplasmic Actomyosin Fibrils in Tissue Culture Cells. Direct Proof of Contractility by Visualization of ATP-Induced Contraction in Fibrils Isolated by Laser Microbeam Dissection, Cell Tissue Res., Vol.166 (1976), pp.427–443.

(2) Katoh, K., Kano, Y., Masuda, M., Onishi, H. and Fujiwara, K., Isolation and Contraction of the Stress Fiber, Mol. Biol. Cell, Vol.9 (1998), pp.1919–1938.

(3) Smilenov, L.B., Mikhailov, A., Pelham, R.J., Jr., Marcantonio, E.E. and Gundersen, G.G., Focal Adhesion Motility Revealed in Stationary Fibroblasts, Science, Vol.286 (1999), pp.1172–1174.

(4) Balaban, N.Q., Schwarz, U.S., Riveline, D., Goichberg, P., Tzur, G., Sabanay, I., Mahalu, D., Safran, S., Bershadsky, A., Addadi, L. and Geiger, B., Force and Focal Adhesion Assembly: A Close Relationship Studied Using Elastic Micropatterned Substrates, Nat. Cell. Biol., Vol.3 (2001), pp.466–472.

(5) Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A. and Burridge, K., Rho-Mediated Contractility Exposes a Cryptic Site in Fibronectin and Induces Fibronectin Matrix Assembly, J. Cell Biol., Vol.141 (1998), pp.539–551.

(6) Coghill, I.D., Brown, S., Cottle, D.L., McGrath, M.J., Robinson, P.A., Nandurkar, H.H., Dyson, J.M. and Christina, A., Mitchell, C.A., FHL3 Is an Actin-Binding Protein that Regulates α-Actinin-Mediated Actin Bundling. FHL3 Localizes to Actin Stress Fibers and Enhances Cell Spreading and Stress Fiber Disassembly, J. Biol. Chem., Vol.278 (2003), pp.24139–24152.

(7) Wojciak-Stothard, B. and Ridley, A.J., Shear Stress-Induced Endotherial Cell Polarization Is Mediated by Rho and Rac but Not Cdc42 or PI 3-Kinases, J. Cell Biol., Vol.161 (2003), pp.429–439.

(8) Noria, S., Xu, F., McCue, S., Jones, M., Gotlieb, A.I. and Langille, B.L., Assembly and Reorientation of Stress Fibers Drives Morphological Changes to Endothelial Cells Exposed to Shear Stress, Am. J. Path., Vol.164 (2004), pp.1211–1223.

(9) Ridley, A.J. and Hall, A., The Small GTP-Binding Protein Rho Regulates the Assembly of Focal Adhesions and Actin Stress Fibers in Response to Growth Factors, Cell, Vol.70 (1992), pp.389–399.

(10) Vouret-Craviari, V., Boquet, P., Pouyssegur, J. and Van Obberghen-Schilling, E., Regulation of the Actin Cytoskeleton by Thrombin in Human Endothelial Cells: Role of Rho Proteins in Endothelial Barrier Function, Mol. Biol. Cell, Vol.9 (1998), pp.2639–2653.

(11) Leung, T., Chen, X.O., Manser, E. and Lim, L., The p160 RhoA-Binding Kinase ROK Alpha Is a Member of a Kinase Family and Is Involved in the Reorganization of the Cytoskeleton, Mol. Cell. Biol., Vol.16 (1996), pp.5313–5327.

(12) Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y. and Kaibuchi, K., Formation of Actin Stress Fibers and Focal Adhesions Enhanced by Rho-Kinase, Science, Vol.275 (1997), pp.1308–1311.

(13) Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K., Regulation of Myosin Phosphatase by Rho and Rho-Associated Kinase (Rho-Kinase), Science, Vol.273 (1996), pp.245–248.

(14) Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K., Phosphorylation and Activation of Myosin by Rho-Associated Kinase (Rho-Kinase), J. Biol. Chem., Vol.271 (1996), pp.20246–20249.

(15) Wanatabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S., Cooperation between mDia1 and ROCK in Rho-Induced Actin Reorganization, Nat. Cell Biol., Vol.1 (1999), pp.136–143.

(16) Wang, Y.-L., Reorganization of Actin Filament Bundles in Living Fibroblasts, J. Cell Biol., Vol.99 (1984), pp.1478–1485.

(17) Ballestrem, C., Wehrle-Haller, B. and Imhof, B.A., Actin Dynamics in Living Mammalian Cells, J. Cell Sci., Vol.111 (1998), pp.1649–1658.

(18) Machesky, L.M. and Hall, A., Role of Actin Polymerization and Adhesion to Extracellular Matrix in Rac- and Rho-Induced Cytoskeletal Reorganization, J. Cell Biol., Vol.138 (1997), pp.913–926.

(19) Chrzanowska-Wodnicka, M. and Burridge, K., Rho-Stimulated Contractility Drives the Formation of Stress Fiber and Focal Adhesions, J. Cell Biol., Vol.133 (1996), pp.1403–1415.

(20) Halliday, N. and Tomasek, J.J., Mechanical Properties of the Extracellular Matrix Influence Fibronectin Fibril Assembly in Vitro, Exp. Cell Res., Vol.217 (1995), pp.109–117.

(21) Mackay, D.J.G., Esch, F., Furthmayr, H. and Hall, A., Rho- and Rac-Dependent Assembly of Focal Adhesion Complexes and Actin Filaments in Permeabilized Fibroblasts: An Essential Role for Ezrin/Radixin/Moesin Proteins, J. Cell Biol., Vol.138 (1997), pp.927–938.

(22) Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S., Calcium Sen-

sitization of Smooth Muscle Mediated by a Rho-Associated Protein Kinase in Hypertension, Nature, Vol.389 (1997), pp.990–994.

(23) Kusumi, A., Sako, Y. and Yamamoto, M., Confined Lateral Diffusion of Membrane Receptors as Studied by Single Particle Tracking (Nanovid Microscopy). Effects of Calcium-Induced Differentiation in Cultured Epithelial Cells, Biophys. J., Vol.65 (1993), pp.2021–2040.

(24) Chan, W.C.W. and Nie, S., Quantum Dot Bioconjugates for Ultrasensitive Nonisotopic Detection, Science, Vol.281 (1998), pp.2016–2018.

(25) Kraft, T., Yu, L.C., Kuhn, H.J. and Brenner, B., Effect of Ca^{2+} on Weak Cross-Bridge Interaction with Actin in the Presence of Adenosine 5′-[γ-thio]triphosphate, Proc. Natl. Acad. Sci. USA., Vol.89 (1992), pp.11362–11366.

(26) Schliwa, M. and Woehlke, G., Molecular Motors, Nature, Vol.422 (2003), pp.759–765.

(27) Umemoto, S. and Sellers, J.R., Charaterization of in Vitro Motility Assays Using Smooth Muscle and Cytoplasmic Myosins, J. Biol. Chem., Vol.265 (1990), pp.14864–14869.

(28) Hall, A., Rho GTPases and the Actin Cytoskeleton, Science, Vol.279 (1998), pp.509–514.

(29) Riveline, D., Zamir, E., Balaban, N.Q., Schwarz, U.S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. and Bershadsky, A.D., Focal Contacts as Mechanosensors: Externally Applied Local Mechanical Force Induces Growth of Focal Contacts by an mDia1-Dependent and ROCK-Independent Mechanism, J. Cell Biol., Vol.153 (2001), pp.1175–1185.