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研究課題名（和文）What is the molecular mechanism of stress fiber disassembly caused by cyclic stretch ?

研究課題名（英文）What is the molecular mechanism of stress fiber disassembly caused by cyclic stretch?

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研究成果の概要（和文）：繰り返し伸展刺激というのは、細胞に対して律動的に伸展・弛緩するプロセスである。細胞に繰り返し伸展刺激を加えると、細胞骨格であるアクチンストレスファイバー(SFs)が脱重合する。この研究には、まず、伸展プロセスではなく、弛緩プロセスがSFsの脱重合に重要であることを証明した。また、従来の研究には、弛緩されたストレスファイバーの脱重合について、アクチン切断タンパク質のcofilinが関与していると考えた。しかし、そこで研究者は、cofilinではなく、ストレスファイバーの一つの構成部分である非筋型ミオシンIIがSFの方向依存脱重合のメカニズムであると考えて検証実験を行った。

研究成果の概要（英文）：Stress fibers (SFs), which consist of actin filaments and nonmuscle myosin II (NMII), undergo disassembly after cyclic stretch (CS) exposure. CS involves cell lengthening and shortening. I found that fast shorteningof cells that exceeded the intrinsic contraction speed of SFs, but not slow shortening and fast/slow lengthening, caused SF disassembly. Since NMII disassociation from actin occurred in cells exposed to a fast shortening process exceeding the rate of the NMII motion, the results of the study demonstrated that NMII should be involved in the disassembly of SFs in cells, which was further confirmed by basic experiments: SFs whose actin-NMII interactions were restricted were more prone to disassembly upon fast cell shortening. On the other hand, SF disassembly still occurred in cells exposed to CS even in cofilin-inactivated cells. Therefore, the results demonstrate that dissociation of NMII from actin bundles is the primary mechanism for CS-induced SF disassembly.

研究分野：医工学

キーワード：Cyclic stretch　Stress fiber disassembly　Nonmuscle myosin II

## １．研究開始当初の背景

Stress fibers (SFs) in nonmuscle cells consist of bundles of actin filaments cross-linked mainly by nonmuscle myosin II (NMII) and α-actinin (Fig. 1). It has been well known that SFs change their orientation in response to cyclic stretch, which occurred as a result of the disassembly of previously existing SFs (1). However, the molecular mechanisms of the SF disassembly remain controversial: Nagayama et al. (2) suggested a compression of SFs to be the major trigger, whereas the other by Tondon et al. (3) claimed the lengthening phase in cyclic stretch to be more essential.

Matsui et al. have suggested that all NMII heads may be unbound from actin due to fast but not slow shortening, which would result in the disassembly of SFs upon the loss of the cross-bridges (4). Decrease in the fraction of myosin heads bound to actin in SFs could lead to the complete detachment of a myosin filament from adjacent actin filaments, resulting in unbundling of intact actin sarcomeric units (5). It has been reported that forward strain (lengthening of SFs) on myosin head kept NMII bound to the actin filaments without little SF disassembly, and in contrast, backward strain on the myosin head (shortening of SFs) increased the ADP release rate, which resulted in short lifetimes of NMII-actin interactions (6). Therefore, I hypothesized that shortening phases, but not lengthening phases of cyclic stretch, induced SF disassembly (Fig. 2). In addition, since rapid SF disassembly was observed only when stress in SFs is relaxed faster than myosin sliding velocity (7), I further hypothesized that disassembly of SFs induced by NMII occurred only when the shortening rate of cyclic stretch was faster than the NMII sliding velocity (< 5. 2% strain/sec) (Fig. 2). Meanwhile, Hayakawa et al. (8) proposed that selective binding of cofilin may be responsible for the disassembly of SFs during cyclic stretch. In this study, I also investigated the role of cofilin in the disassembly of SFs.

## ２．研究の目的

(1) To specify the effects of lengthening/shortening phases of cyclic stretch on SF disassembly. I would like to test the hypothesis that SF disassembly was dependent on the rate of shortening.

(2) To confirm the molecule mechanisms responsible for the SF disassembly exposed to cyclic stretch. I would like to prove that NMII, but not cofilin was essential for cyclic stretch-induced SF disassembly.

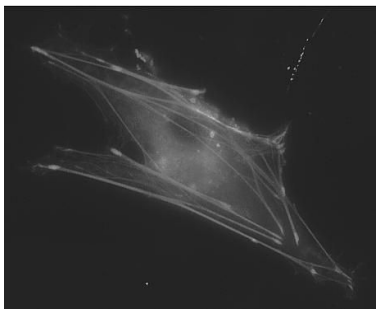

Fig. 1 SFs in a cell

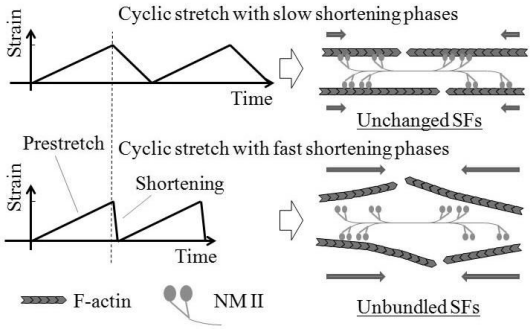


Fig. 2 Hypothesis of the current study that explains the disassembly (unbundling) process of SFs

## ３．研究の方法

### 3.1 Cell culture

For the experiments, non-muscle cells including primary bovine artery smooth muscle cells (BASMs) (Cell Applications) and mouse U2OS osteosarcoma cell line were used. Cells were cultured with low glucose (BASMs) or high glucose (U2OS cell line) Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (JRH Bioscience) and 1% penicillin-streptomycin (Invitrogen) in an incubator at 37°C and 5% $CO_2$/95% air. BASMs were used for experiments from 5th to 12th generations.

### 3.2 Transfection of plasmids into U2OS cell line

U2OS cells were removed from the culture dish with 0.05% trypsin-EDTA and seeded on a 4 $cm^2$ stretch chamber coated with 10 μg/ml fibronectin from bovine (Sigma Aldrich) under sparse conditions ($5\times10^4$ cells/$cm^2$) and pre-cultured statically for 24 h.

In the experiment taking live-cell images of U2OS cells exposed to shortening- or lengthening-specific cyclic stretch, a plasmid encoding enhanced yellow fluorescent protein (EYFP)-actin was transfected into U2OS cells for visualization of actin cytoskeleton.

Since myosins (both smooth muscle and non-muscle) are regulated by the phosphorylation

of MCL-2 (9), to evaluate the causal role of myosin activation in the actin stress fiber (SF) disassembly, U2OS cells were transfected with plasmid vectors 24 h prior to experiments, which express enhanced GFP-tagged wild type MLC-2 (WT-MLC-2-EGFP), MLC-2-DD-EGFP, MLC-2-AS-EGFP, and MLC-2-AA-EGFP. MLC-2-DD-EGFP is a phosphomimetic MLC mutant, in which MLC-2 phosphorylation was mimicked at Thr18 and Ser19. On the other hand, transfection with MLC-2-AS-EGFP led to enhancement of phosphorylation at Ser19 and inhibition of phosphorylation at Thr18, respectively. The other plasmid, MLC-2-AA-EGFP, is a dominant negative mutant of MLC-2, in which Thr18 and Ser19 residues were changed to alanine and phosphorylation at Thr18 and Ser19 was inhibited.

In order to check whether or not cofilin plays a primary role in the SF disassembly in cells exposed to cyclic stretch, U2OS cells were transfected with plasmid DNA 12 h before the experiments to regulate the phosphorylation of cofilin. As LIM-kinase is known to be a serine/threonine kinase that phosphorylates and inactivates cofilin (10), I transfected cells with Myc- or HA-tagged wild type (WT), dominant negative (DN), or constitutively active LIM-kinase (CA). DN and CA were supposed to up-regulate and down-regulate activated cofilin, respectively.

All of the transfection in this study was performed using a liposomal method with the invitrogen transfection reagent (invitrogen) according to the manufacture's protocol.

### 3.3 Creation of shortening- and lengthening-specific cyclic stretch

It has been reported that a 20%, 0.5 Hz cyclic stretch (1-sec stretch and then 1-sec shortening for each cycle) induced the disassembly and orientation change of SFs in cells (11). In this study, to specify the effects of shortening or lengthening phase independently, cyclic stretch with the asymmetric strain waveforms were applied to cells. Strain waveform with 20-sec lengthening and 1-sec shortening was referred as fast shortening cyclic stretch, and the asymmetric strain waveform with 1-sec lengthening and 20-sec shortening was referred as fast lengthening cyclic stretch (Fig. 3a, c). In addition, to confirm the rate-dependency, I also applied asymmetric waveforms with 10-sec lengthening (slow lengthening cyclic stretch) or 10-sec shortening (slow shortening cyclic stretch), with a 20-sec shortening/lengthening in the opposite phase of the cycle (Fig. 3b, d).

### 3.4 Application of cyclic stretch to cells

In order to examine the rate dependency of shortening-specific cyclic stretch on the disassembly of actin stress fibers (SFs) in cells exposed to cyclic stretch, I applied patterned BASMs in this study. SFs in BASMs consist of bundles of actin filaments cross-linked by non-muscle myosin II (NMII) and the alignment of SFs could be pre-determined on stretch chambers using a micro-patterning technique, as reported by Deguchi et al. (12). Cells are constrained onto micro-islands with a 25-μm width in stretch chambers, the direction of which is parallel to the axis of cyclic stretch. After the confirmation of the formation of cell micro-islands using a phase-contrast microscopy (Olympus), the stretch chamber was subjected to fast shortening cyclic stretch for 10 and 30 minutes, or slow shortening cyclic stretch for 10, 30, and 43 minutes, respectively.

In order to confirm the results from experiments using patterned BASMs, I also observed live U2OS cells using the live cell imaging technique. For live cell imaging, YFP-actin-transfected cells in a stretch chamber were placed inside a 37°C stage-top incubator with 5% $CO_2$ humidified air, and subjected to 10% cyclic stretch with the asymmetric strain waveforms described above. Observation of live cells was performed on an inverted confocal microscope (IX71, Olympus), and time-lapse images of live cells were collected every 5 minutes for 2 h.

On the other hand, cyclic stretch experiment using U2OS cells transfected with LIM-kinase or myosin II mutants was performed in an incubator at a temperature of 37°C. The strain waveform with a fast shortening rate (a 1-s shortening rate) (Fig. 3a) was applied to cells transfected with LIM-kinase or myosin II mutant.

### 3.5 Analysis of SF disassembly

In some experiments, cells were fixed and subjected to immunofluorescence staining. In order to calculate the extent of SF disassembly, an image processing algorithm based on pixel intensity gradients used in the previous study was applied (11, 13, 14).

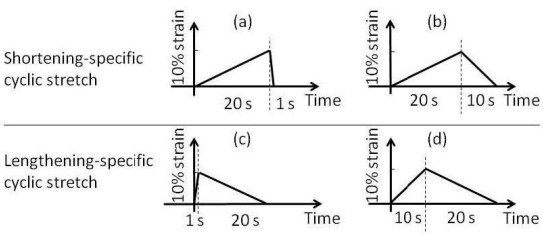


Fig. 3 Strain waveforms used in this study

## ４．研究成果

### 4.1 Fast shortening was essential for the disassembly of SFs in cells exposed to cyclic stretch

Uni-directional alignment of SFs in BASMs on the patterned membrane of stretch chamber was confirmed. Both static cells and cells exposed to cyclic stretch with 10 sec-shortening phases for 10 or 30 min had well-defined straight line-shaped SFs. However, in cells exposed to cyclic stretch with 1 sec-shortening phases for 10 or 30 min, most of SFs were disrupted. Living cells exposed to cyclic stretch with 10 sec-shortening phases, 1 sec- and 10 sec-lengthening phases, still had well-defined straight line-shaped SFs after 2 h-cyclic stretch exposure. In contrast, previously existing SFs in cells exposed to 1 sec-shortening cyclic stretch were disrupted and the obvious change could be observed after 30-min cyclic stretch exposure.

### 4.2 Results of overexpression or suppression of cofilin activity

As only 1 sec-shortening cyclic stretch led to SF disassembly in cells exposed to cyclic stretch, we applied the same shortening rate to cells transfected with LIM-kinase mutant. SFs in cells transfected with WT or DN for enhanced cofilin activity were similar to those in non-transfected cells under static conditions, and after exposure to cyclic stretch for 2 h, SFs roughly oriented perpendicular to the direction of stretch. On the other hand, part of SFs in CA-transfected cells, in which cofilin activity was down-regulated, were slightly disrupted under static conditions, and I found that SFs in CA-transfected cells subjected to cyclic stretch disassembled more profoundly, without a re-assembly in the direction perpendicular to the direction of stretch after 2 h.

### 4.3 Results of experiments with cells transfected with DNA for the regulation of NMII activity

Cells transfected with MLC-2-DD-EGFP, in which phosphorylation was mimicked at 18 and 19, showed little evidence of stress fiber disruption compared with control cells. Cells transfected with MLC-2-AS-EGFP or MLC-2-AA-EGFP, in which the actin- myosin II interactions was restricted, had stress fibers highly disrupted, with a fuzzy diffuse appearance after cyclic stretch exposure compared to control cells. In addition, most stress fibers were highly disrupted or absent in cells transfected with MLC-2-AA-EGFP after cyclic stretch exposure.

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**〔産業財産権〕（計１件）**

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