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High extensibility of stress fibers revealed by in vitro micromanipulation with fluorescence imaging

Tsubasa S. Matsui [a], Masaaki Sato [b,c], Shinji Deguchi [c,*]

[a] Department of Biomolecular Sciences, Tohoku University, Japan
[b] Department of Biomedical Engineering, Tohoku University, Japan
[c] Department of Bioengineering and Robotics, Tohoku University, Japan

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ABSTRACT

Stress fibers (SFs), subcellular bundles of actin and myosin filaments, are physically connected at their ends to cell adhesions. The intracellular force transmitted via SFs plays an essential role in cell adhesion regulation and downstream signaling. However, biophysical properties intrinsic to individual SFs remain poorly understood partly because SFs are surrounded by other cytoplasmic components that restrict the deformation of the embedded materials. To characterize their inherent properties independent of other structural components, we isolated SFs from vascular smooth muscle cells and mechanically stretched them by in vitro manipulation while visualizing strain with fluorescent quantum dots attached along their length. SFs were elongated along their entire length, with the length being approximately 4-fold of the stress-free length. This surprisingly high extensibility was beyond that explained by the tandem connection of actin filaments and myosin II bipolar filaments present in SFs, thus suggesting the involvement of other structural components in their passive biophysical properties.

1. Introduction

Stress fibers (SFs) consist of actin bundles cross-linked mainly by smooth muscle and nonmuscle myosin II [1]. SFs are physically connected at their ends to cell–substrate adhesions (or focal adhesions, FAs) via integrin, and as a subcellular contractile apparatus, they exert tension on the adhesions. It has become increasingly clear that myosin-generated tension is critical for maintaining the structure and function of FAs, which in turn regulates downstream signaling for cell growth, differentiation, and death [2,3].

To evaluate the role of SFs as a tension-exerting component, the knowledge of their mechanical properties is important. In order to determine their passive mechanical properties or load-bearing ability, atomic force microscopy has been used to stretch a part of the lateral side of SFs within cells [4,5]. However, interpretation of these data may be generally complicated as SFs are surrounded by other structural components in the cytoplasm that should restrict the deformation of the embedded SFs of interest. Thus, the mechanical properties intrinsic to individual SFs remain unclear.

In our earlier studies [6–8], we extracted SFs from cultured cells and isolated them from the substrate. The use of isolated SFs allows characterization of their inherent properties, independent of the surrounding structural materials. With micromanipulation in the axial direction, we showed that isolated SFs are highly extensible in the "rigor" state where ATP is absent. However, we did not observe local strain during stretching. Thus, we could not rule out the possibility that the high extensibility might be caused by a tear at a particular region of the isolated SFs and the rest of the regions remained undeformed.

In the present study, we provide the first direct evidence that rigor SFs are indeed extensible with observation of local strain during in vitro micromanipulation. We used rigor SFs (with no cyclic actin–myosin cross-bridging) for the experiments on the basis of our assumption that SFs may work substantially with low ATP consumption during physiological stretching of cells. More specifically, recent studies that measured kinetics [9] or single molecule dynamics [10] have demonstrated that smooth muscle and nonmuscle myosin II subjected to resistive loading tend to attach to actin for a fairly long time because their ADP-release rate is significantly reduced upon external loading. Many myosin II filaments within tension-bearing SFs may therefore remain attached to actin for a relatively long time given that myosin II is constantly subjected to resistive loading in the tension-bearing SFs. Thus, SFs bearing cellular tension could behave similarly to those in the rigor state.

* Corresponding author. Present address: Department of Nanopharmaceutical Sciences, Nagoya Institute of Technology, Gokiso, Showa, Nagoya 466-8555, Japan.
E-mail address: deguchi@nitech.ac.jp (S. Deguchi).

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2. Results

2.1. Evaluation of intactness of extracted SFs

SFs were extracted from cells to remove other cytoplasmic constituents including intracellular ATP that induces the actin–myosin interaction. To evaluate the intactness of the extracted SFs, we observed their sarcomeric structure and contractility. In this experiment, SFs were extracted from cells transiently expressing GFP-α-actinin. Semi-periodic dotted patterns of sarcomeres visualized with α-actinin were observed along the length of the extracted SFs, similar to those within the cells (Fig. 1). Thus, the sarcomere structure was maintained even in the extracted SFs, at least at the macroscopic scale. Reactivation solution containing 1 mM ATP was added to SFs. Subsequent gradual shortening of SFs themselves as well as of the sarcomere distances were observed, thus demonstrating that extracted SFs retain intactness in terms of ATP-dependent contractility.

2.2. Stretching of isolated SFs independent of surrounding materials

SFs were extracted from cells and labeled with QDs–phalloidin. Using a pair of needles, a single SF was physically scraped off the dish and then stretched quasi-statically within the horizontal

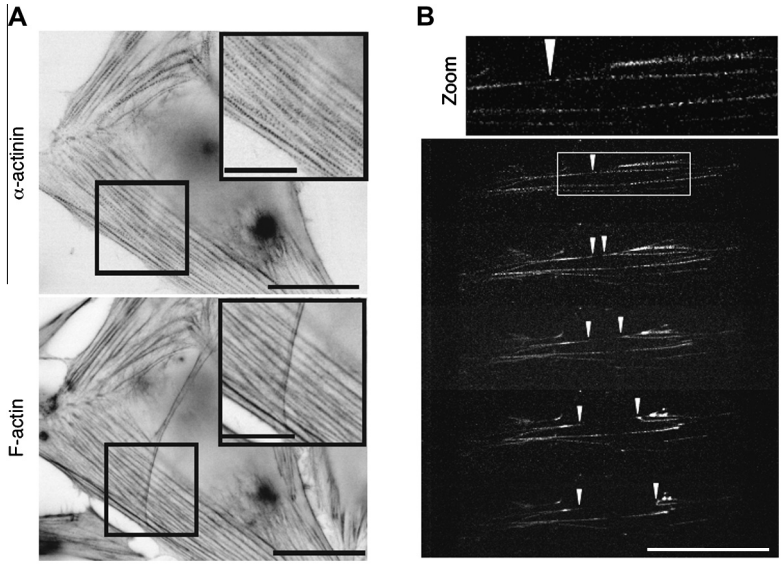

Fig. 1. Maintained sarcomeres and contractility of extracted SFs. (A) Sarcomeric patterns observed in cells expressing GFP-α-actinin along the length of SFs shown with fluorescent phalloidin. (B) Sarcomeric patterns of GFP-α-actinin observed in SFs extracted from cells. Addition of 1 mM ATP induced shortening of SFs due to contractility. Scale, 20 μm (A); 10 μm, inset in (A); 50 μm (B).

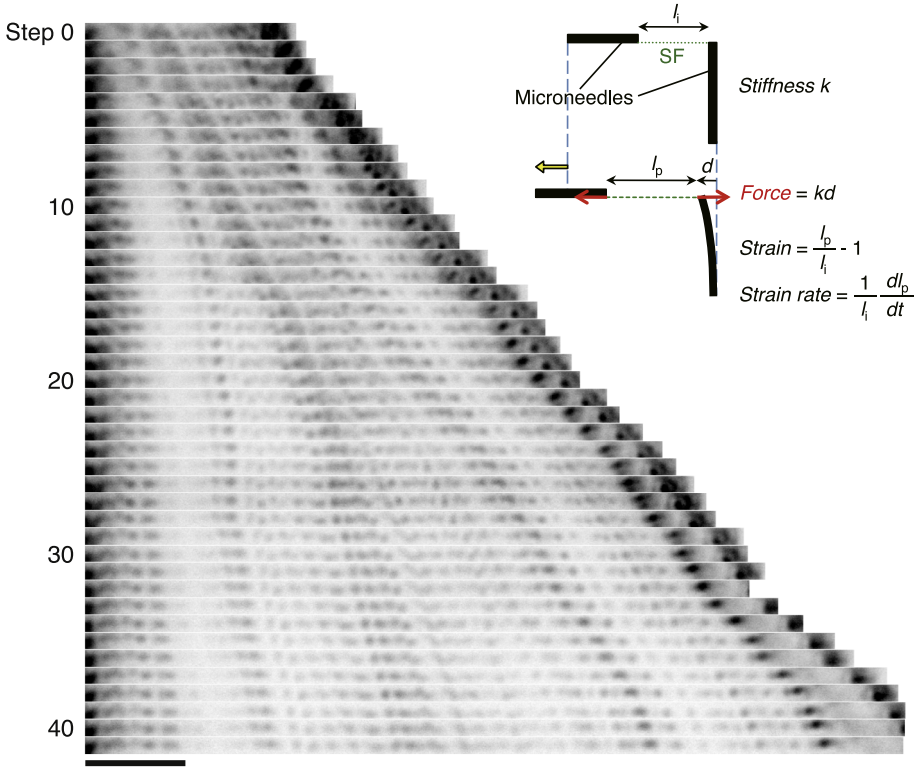

Fig. 2. Kymograph of QDs-phalloidin along the length of a single SF subjected to stretching. Scale, 5 μm. The inset represents a sketch of stretching test of an individual SF.

plane while the fluorescence from QDs along its length was observed (Fig. 2). The total stretch ratio (the current length divided by the initial length) reached approximately 4-fold, stretching from 9.2 to 38 μm in this example.

QDs–phalloidin patterns along the SF length were initially elusive for characterization, whereas semi-periodic patterns appeared gradually during a stretch. With a further stretch, the pattern distances were enlarged in a heterogeneous manner. The local stretch ratio reached 6-fold at the largest and 2.5-fold at the smallest (Fig. 3), and SF was finally detached from the needle tip. For technical reasons, we could not simultaneously measure the force required for this stretching and give precedence to securing the high-strain stretch. Instead, in a separate set of experiments, we measured the force–strain relationship at a lower strain range of 40% (1.4-fold) stretch at strain rates of 0.1 and 0.01 s^{-1} ($n = 6$). The force increased with strain and was on the order of 1–10 nN in magnitude (Fig. 4).

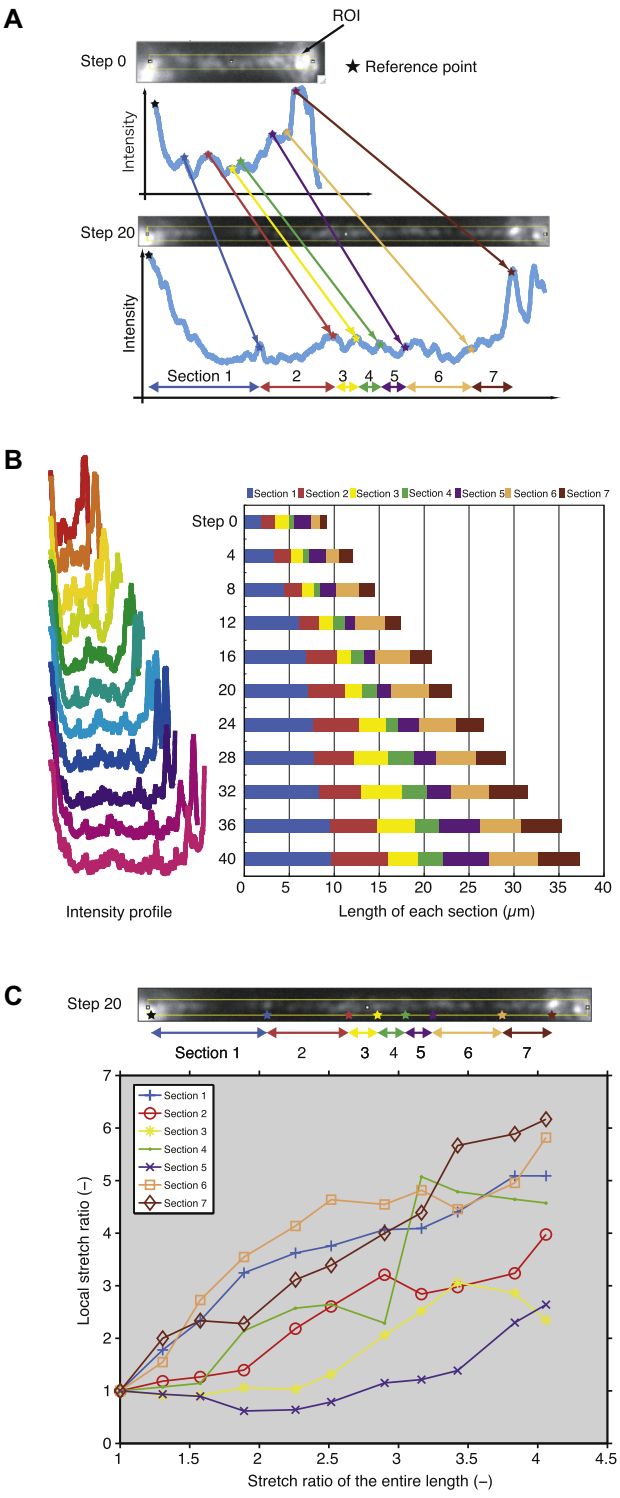

Fig. 3. Strain analysis based on QD patterns. (A) Reference points detected from the fluorescence intensity profile. (B) Time course changes in the intensity profile and the distances between each section. (C) The relationship between the stretch ratios for each section and for the entire length.

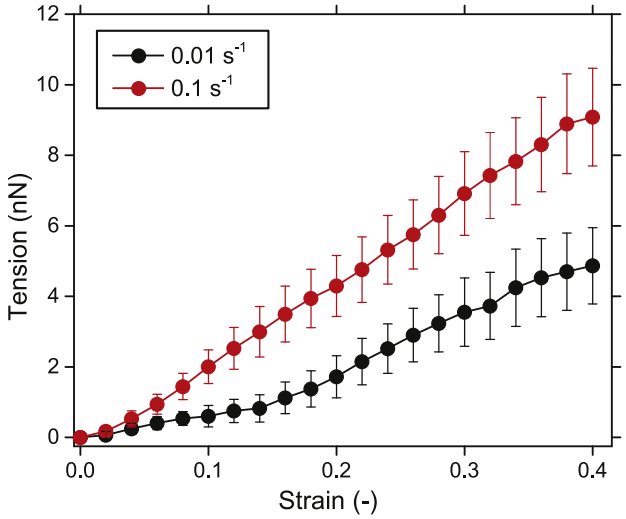

Fig. 4. Force–strain relationship measured at a constant strain rate of 0.1 or 0.01 s^{-1}. Data are expressed as mean ± SEM.

3. Discussion

The high extensibility of single SF shown in this study is consistent with previous results [7] in which the total stretch ratio reached a similar maximum value of 3.75-fold of the initial length. However, in these previous studies, the strain was evaluated by measuring the change in the distance between the two needles used for manipulation. Thus, the macroscopic evaluation of strain did not reveal the origin of the high extensibility. In contrast, the present analysis of QDs-labeled actin filaments provided direct evidence that SFs are highly extensible along their entire length (Figs. 2 and 3).

As mentioned in the Introduction, we contemplated that use of rigor SFs for these experiments would be valid because of the external loading-induced decrease in ADP-release rate in the actin–myosin interaction cycle and consequent long lifetime of their binding. In addition, if we focus only on extensibility, the presence or absence of ATP would make little difference in a high-strain region. Specifically, the magnitude of force we applied was on the order of 10 nN (Fig. 4). Electron microscopy results by Cramer et al. [11] suggested that individual SFs contain approximately 10–30 actin filaments across the width of observed thin-sections. According to the paper, the thickness of the thin-sections was 40 nm, and the diameter of the SF observed was 360 nm. Thus, individual SFs would be composed of 90–270 (=10 × 360/40–30 × 360/40) actin filaments bundled in parallel. Assuming that actin filaments are the major structural components, each actin filament should bear a tension of 0.1–0.04 (=10/90–10/270) nN. The number of myosin heads at each end of the bipolar filament is approximately 10 [9,12–14]; thus, if all the heads are binding to the same actin filament, a force of 10–4 (=0.1/10–0.04/10) pN should be exerted on

each of the actin–myosin cross-bridges. This rough estimate is higher in magnitude than, or at least should be comparable with, that required for breaking skeletal, smooth muscle, or nonmuscle actin–myosin bonds, less than 10 pN [10,15,16]. It is thus likely that actin–myosin bonds subjected to such large tension would eventually be broken even in the rigor state.

The above discussion was consistent with the fact that many of the distances between QDs-labeled actin filaments were finally enlarged tomore than 0.28–0.33 μm (Fig. 3), a reported length of myosin II bipolar filaments that tie different actin filaments [17,18]. Thus, the origin of the high extensibility obtained in this study is beyond that explained by tandemly arranged actin and myosin II filaments.

Cellular titin has been reported to be present along SF length in nonmuscle cells [19,20], but its role in the mechanics of SFs remains unclear. Given the extraordinary extensibility of conventional muscle titin (also known as connectin) that mechanically links thick myosin filaments to thin actin filaments via the Z-disc in the muscle sarcomere [21,22], it is reasonable to speculate that the nonmuscle isoform may function as a passive stress-bearing component within SFs. Thus, experiments for identifying the involvement of cellular titin in the mechanical properties of SFs should be the subject of future investigation.

In summary, by manipulating isolated but functional (in terms of the maintenance of sarcomere patterns and contractility) SFs, we demonstrated that they can be stretched to approximately 4-fold of the stress-free length. This extensibility is beyond that explained by the tandem connection of actin filaments and myosin II bipolar filaments, thus suggesting the involvement of other structural components in their passive mechanical properties.

4. Materials and methods

4.1. Cell culture and transfection

Primary bovine aortic smooth muscle cells (Cell Applications) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% each of penicillin and streptomycin. The cells were maintained in a 5% CO_2 incubator at 37°C. For fluorescence imaging, the cells were transfected with plasmids expressing GFP-α-actinin (a gift from Dr. Imamura, National Center of Neurology and Psychiatry, Japan) using Lipofectamine (Invitrogen), according to the manufacturer's directions.

4.2. Extraction of SFs

SFs were extracted from cells seeded on a glass-bottom dish following Katoh et al. [23] with modifications. The following procedures were performed on ice. The cells were subjected to hypotonic treatment with 2.5 mM triethanolamine in distilled water for 30 min and then to treatment with 50% glycerol in PBS for 5 min. SFs extracted at this stage were washed with a cytoskeleton-stabilizing buffer (10 mM imidazole, 100 mM KCl, and 2 mM EGTA) for 10 min. All solutions used were supplemented in advance with 1 μg/ml pepstatin and 1 μg/ml leupeptin. In separate experiments, intactness of extracted SFs was evaluated to determine the maintenance of sarcomeres and contractility. To observe sarcomeres, the cells transiently expressing GFP-α-actinin were fixed with 4% paraformaldehyde and stained with Alexa-546-phalloidin–QDs for F-actin staining. In a separate experiment, SFs were extracted from cells expressing GFP-α-actinin and then they were treated on a microscope (IX-71; Olympus) with reactivation solution containing 1 mM ATP (3 mM $MgCl_2$, 75 mM KCl, 20 mM imidazole, and 1 mM EGTA) to induce contraction in vitro.

4.3. Stretching of isolated SFs

For visualizing actin filaments, the extracted SFs were treated with 0.33 μM biotin–phalloidin (Invitrogen) in PBS for 15 min and washed with PBS and then with 1.6 nM streptavidin–QD655 (Quantum Dot Corporation) in PBS containing 1% BSA for 5 min at room temperature. The extracted SFs were then isolated from the dish using a rubber scraper and were immersed in a 35-mm glass-bottom dish with the cytoskeleton-stabilizing buffer containing 2.3 mg/ml glucose, 0.018 mg/ml catalase, and 0.1 mg/ml glucose oxidase for oxygen removal. Two glass needles (approximately 1 μm in diameter at the tips) were used for capturing individual SFs. The needles and SFs were observed through an objective lens (UPlanApo 100× oil, Iris 3, NA 1.30; Olympus). The tip of the needles was coated with epoxy resin immediately prior to attaching SFs. Single SF was caught between the needle tips and was lifted up approximately 10 μm from the dish bottom. After been held in position for 20 min, we displaced the parallel needle outward quasi-statically at a step length of 1 μm to stretch SFs. QD-labeled F-actin on the stretched individual SFs was imaged using the camera at each step. Images were analyzed using ImageJ (NIH).

4.4. Force measurement

The force required for stretching SFs was measured using a home-made device described elsewhere in detail [14]. Here we prepared two fine glass needles, one of which is flexible and used as a force sensor, and the other was adjustably positioned using a piezo-actuator. Single SF was held between the needle tips, with the flexible one placed perpendicular to its axis and the other parallel to it. SF was stretched by moving the parallel needle outward to give a constant strain rate of 0.1 or 0.01 s^{-1} that was realized with a visual feedback control.

Acknowledgments

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