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Mechano-sensing by actin filaments and focal adhesion proteins

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Mechanosensitive ion channels have long been the only established molecular class of cell mechanosensors with known molecular entities. However, recent advances in the state-of-the-art techniques, including single-molecule manipulation and imaging, have enabled an investigation of non-channel type cell mechanosensors and the underlying biophysical mechanisms of their activation. To date, two focal adhesion proteins, talin and p130Cas, have been postulated to act as putative mechanosensors, acting through mechano-induced unfolding of their particular soft domain(s) susceptible to phosphorylation. More recently, the actin filament has been demonstrated to act as a mechanosensor in the presence of the soluble actin-severing protein, cofilin. The cofilin severing activity negatively depends on the tension in the actin filament through tension-dependent binding/unbinding of cofilin to/from the actin filament. As a result, relaxed actin filaments are severed, while tensed ones are either not severed or severed after a long delay. Here we review the latest progress in the mechanosensing by non-channel type proteins and discuss the possible physiological roles of the mechanosensing performed by actin filaments in the course of cell migration.

Introduction: Stress Concentration Sites and Mechanosensing in the Cell

The hundred trillions of cells which make up our bodies are continually exposed to various mechanical stimuli, including muscle contraction, ongoing blood flow, blood pressure, distension of visceral organs, etc., which initiate a wide range of cellular responses. These responses include Ca^{2+} mobilization,[1,2] protein phosphorylation,[3-5] rearrangement of the cytoskeleton,[6,7] transcriptional regulation,[8] apoptotic cell death,[9,10] and cell differentiation[11] and so on. Mechanical forces are sensed by mechanosensors that presumably undergo change in their enzymatic activity or interaction with signaling molecules in response to forces. However, the particular molecular entities and the underlying biophysical mechanisms of these mechanosensing molecules are largely unknown except for the mechanosensitive (MS) channels.[12-14] A major reason for this slow progress is that mechanosensors, by their nature, do not possess the specific chemical ligands such as agonists, antagonists and inhibitors, which are used as biochemical tools to detect and purify receptor molecules. An alternative way toward the molecular identification of mechanosensors relies on an idea that putative mechanosensors are most likely localized at cellular sites of high concentrations of stress. The cell membrane is such a structure due to its high lateral elastic modulus, and is actually endowed with MS channels, although the correlation between the highly stressed membrane region and the MS channel localization has yet to be demonstrated. Here we focus on adhesive structures, including focal adhesions, the actin cytoskeleton, and the molecular apparatus connecting these structures, where stress is presumably highly concentrated.

Generally focal adhesions comprise a high stress concentration site, linking extracellular matrices and the actin cytoskeleton.[15] Mechanical forces imposed from inside or outside of the cell are transmitted through the focal adhesions bidirectionally, i.e., in an outside-in or inside-out direction.[16] Exogenous mechanical forces are exerted on integrins, an extracellular matrix receptor enriched in focal adhesions that activate a variety of intracellular signaling cascades.[17-19] The activities of actin modulating proteins[20] are also influenced by endogenous cell contractile force[21] or exogenous mechanical stimuli.[6,7] Thus, mechanosensors[22] and directly associated signaling molecules[15] are thought to be involved in the focal adhesion, the actin cytoskeleton and/or cellular structures linking focal adhesions and the actin cytoskeleton.

A recent in vitro biophysical study has shown that the apparent actin filament severing activity of cofilin and the rates of binding of cofilin to actin filaments are both affected by the tension present in the actin filaments,[23] implying that the actin filament itself works as a mechanosensor.

Here, we review the recent progress in the study of tension-sensing by focal adhesion proteins and actin filaments, and evaluate the possible physiological roles of such tension-sensing by actin filaments.

Tension Sensing by Focal Adhesion Proteins

Mounting evidence suggests that focal adhesion proteins are involved in the mechanically triggered activation of intracellular signaling molecules,[15] including MAP kinases,[24] Akt,[25,26] and PI3 kinase.[27] Direct manipulation and imaging of single protein molecules enables an application of mechanical forces to a target protein while monitoring its response, using these methods

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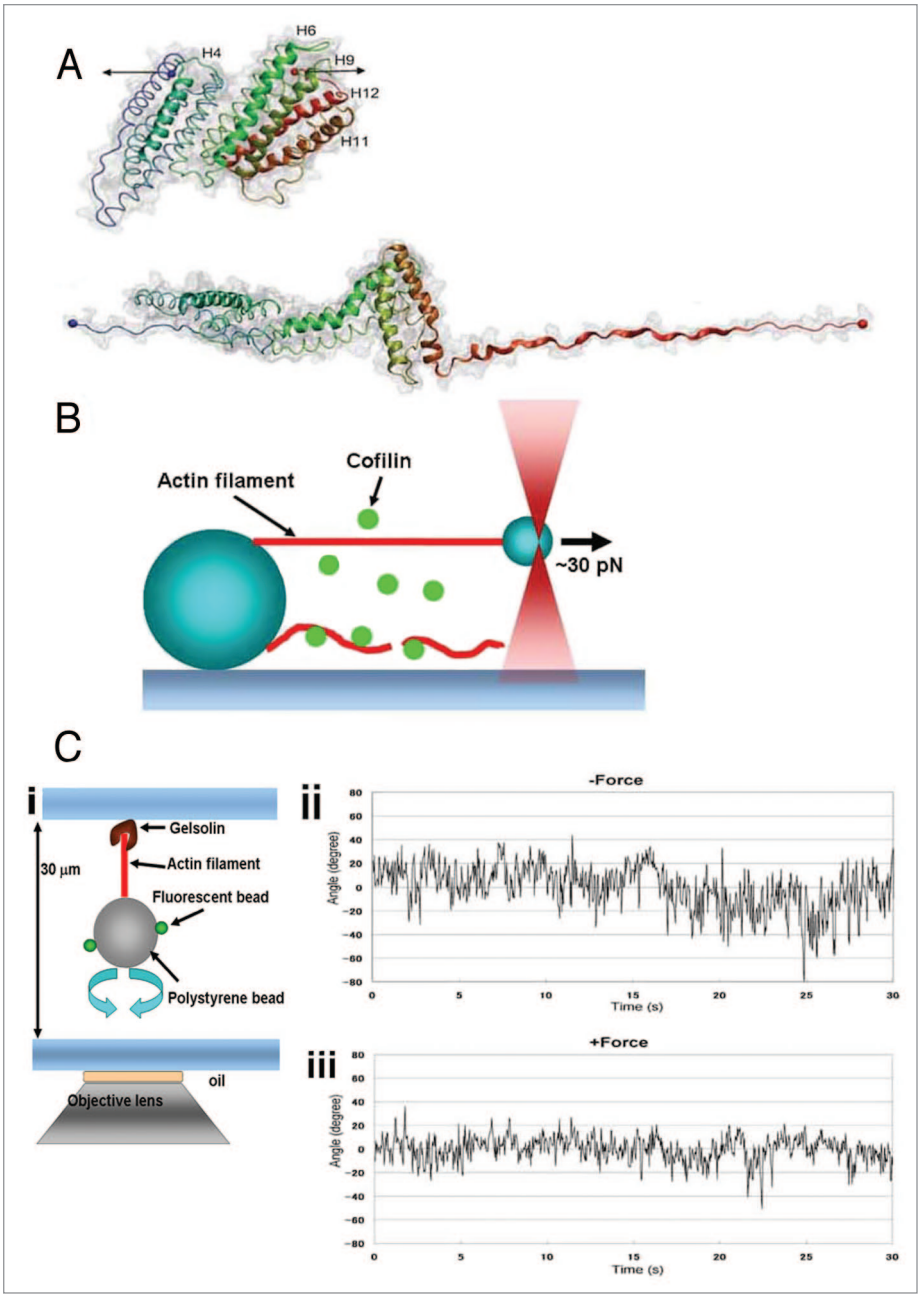

Figure 1. (A) Structure of the talin rod domain consisting of 12 helices (upper panel). The rod domain which is unfolded under force[28] (lower panel) is shown by the black arrows in the upper panel. **(B)** Schematic drawing of the experimental setup to apply force to a single actin filament.[23] One end of an actin filament (red) was tethered to a bead fixed on a coverslip, and the other end of the filament was tethered to a small bead manipulated by optical tweezers. The lower actin filament is not tensed, and is severed by cofilin. **(C) (i)**, Schematic drawing of the experimental setup to trace the torsional fluctuations of a single actin filament. An actin filament is tethered on the coverslip via gelsolin, and a bead was attached to the lower end of the actin filament. Rotation of the bead is monitored using fluorescent small beads that are attached on the large bead. **(ii)**, Rotational angular fluctuations of a bead attached to an actin filament during the time the large bead was trapped, but not stretched, by optical tweezers. **(iii)**. The rotational angular fluctuations were decreased when the actin filament was stretched by moving the trapping point in downward direction. The data show the results at zero and ca. 5 pN stretch force; one can change the applied force by increasing the laser power of the optical tweezers. Panels A and C are based on studies (ref. 28) and (ref. 23), respectively.

talin[28] and possibly p130Cas[29] have been proposed to work as a mechanosensor.

The focal adhesion protein talin has a head domain that binds to the cytoplasmic tail of the integrin β subunit, while its rod domain contains actin-binding sites, each of which locates adjacent to a vinculin-binding site. When mechanical force was applied to talin through the manipulation of a small bead attached on a focal adhesion, integrin-cytoskeleton linkage at the focal adhesion was strengthened[30] and vinculin was translocated to the focal adhesion underneath the bead in vivo,[31] suggesting that talin acts as a mechanosensor in focal adhesions.[30,32]

A recent in vitro study demonstrated tension-sensing by the talin rod domain. The recombinant talin rod domain was extended approximately 100 nm by direct application of force (20 pN)[28] with an AFM tip (**Fig. 1A**), resulting in an increase in the number of vinculin head domains bound to the talin rod domain. Combining molecular and cellular level studies, the authors have proposed that the force applied through the integrins in the focal adhesions extends the talin rod domain and exposes its binding sites for vinculin, which reinforces the actin-integrin linkage in vivo.

p130Cas, a substrate for p60Src,[29] is a scaffolding protein that localizes at focal adhesions. Stretching of the cell substrate induces tyrosine phosphorylation of p130Cas, followed by the activation of the p38 MAP kinase cascade via small GTPase Rap1[33] in intact cells, suggesting that p130Cas is involved in mechanically-induced signal transduction. A recent biophysical study using the purified p130cas substrate domain for p60Src suggested that p130Cas might work as a mechanosensor. Mechanical extension of the elastic substrate, to which the purified recombinant p130Cas substrate domain is adhered, induces tyrosine phosphorylation of the p130Cas substrate domain by p60Src, implying that the mechanical unfolding of the p130Cas substrate domain increases the phosphorylation level of the p130Cas not only in vitro but also in vivo.[34] However, further studies are needed to confirm this phenomenon actually takes place in live cells.

In intact spreading cells, the staining pattern made by an antibody against the extended p130Cas substrate domain shows a similar staining pattern to that made by an antibody against phosphorylated p130Cas in the cell periphery,[34] suggesting that the p130Cas substrate domain is extended by force, and tyrosine-phosphorylated by p60Src.[33] In these mechanosensing processes, the unfolding of the sensor molecules (talin and p130Cas) is presumably required to sense forces and to transmit them to the downstream signaling molecules.

Tension Sensing by Titin and Fibronectin

Titin, a giant elastic muscle protein connecting the Z-disc and M-line in the sarcomere, is unfolded by force in its kinase domain.[35] The improper unfolding of the kinase domain is thought to be involved in muscle disuse atrophy.[36] Fibronectin, an extracellular matrix protein, is mechanically unfolded by cell contractile forces,[37] and the unfolding may be involved in accelerated fibronectin assembly, resulting in an enhancement of the fibronectin-integrin linkage.[38,39]

Tension Sensing by Actin Filaments

A recent in vitro study revealed that the actin filament itself functions as a mechanosensor.[23] One end of a single actin filament was tethered to a myosin-coated bead fixed on a coverslip, while the other end was tethered to a small myosin coated bead manipulated with optical tweezers so as to tense the filament (**Fig. 1B**). When the filament was tensed (~30 pN), it was severed by cofilin with a larger delay compared with the filament when it was not tensed, or was not severed within the observation period (ca. 30 sec). Additionally, the binding of cofilin to the bundles of actin filaments was imaged with fluorescein labeled cofilin, which showed that the rate of the binding of cofilin to the actin bundles decreased when the bundles were tensed. Approximately 2 pN of force is sufficient to decrease the apparent severing activity of cofilin,[23] which is comparable to the force generated by a single myosin head. A single actin stress fiber is composed of 10–30 actin filaments,[40] and the contractile force in a single stress fiber is estimated to be on the order of nN,[41] suggesting that the contractile force in stress fibers (> 2 pN for each actin filament, assuming the stress in the stress fiber is evenly distributed among the actin filaments) is high enough to prevent cofilin from binding to the actin filaments in vivo.

How does tension prevent the binding of cofilin to the actin filaments? There are enormous numbers of biochemical,[42,43] structural,[44,45] and computational[46] studies on the binding of cofilin to actin filaments. Electron microscopic analyses revealed that the twist of the actin filament is increased when the filament is fully decorated by cofilin.[44,45] On the other hand, the rotational angular conformation of actin protomers in native actin filaments is variable;[47] the angle between neighboring actin protomers reportedly ranges from 156° to 170°.[45] Based on these observations, it is hypothesized that cofilin preferentially binds to actin filaments in solution when the protomers of the actin filament are in the twisted state; i.e., large torsional fluctuations are required for the binding of cofilin to the filament.

The torsional fluctuations of single actin filaments were visualized by monitoring the rotation of a bead attached on one end of the filament (**Fig. 1C**). Application of a force of approximately 5 pN reduced the torsional fluctuations of the filament (**Fig. 1C**), indicating that the actin filament fluctuates less when the filaments are tensed, supporting the hypothesis that tension in the actin filament reduces torsional fluctuations of the actin filament, which decreases the effective number of cofilin binding sites so as to prevent the binding of cofilin, resulting in an inhibition of the cofilin severing of the filament. This may constitute the potential tension sensing mechanism performed by actin filaments. Here, the sensor molecule actin filament senses the applied force and transduces it into changes in the fiber fluctuation that in turn modulates the activity (binding here) of the signaling molecule cofilin, eventually regulating the fiber dynamics by itself. This forms a kind of very smart and reliable local feedback regulatory system.

Possible Roles of Tension Sensing by Actin Filaments in Cells Responding to Mechanical Forces

The tension-dependent local disassembly of actin filaments by cofilin presumably works under certain specific physiological conditions. The distribution of cofilin in living cells was examined using a GFP-cofilin fusion protein. GFP-cofilin translocated to the stress fibers within a period of one minute when tension in the stress fibers was reduced by relaxing the pre-stretched elastic cell substratum (**Fig. 2A**), followed by disassembly of the stress fibers,[23] suggesting that cofilin mediates disassembly of the stress fibers with a decline in tension in living cells. BDM, a myosin ATPase inhibitor, reduces tension in the stress fibers, and induces stress fiber disassembly in living cells. Stress fiber disassembly by BDM is reportedly enhanced in cells overexpressing cofilin.[21] By contrast, stress fiber disassembly was not detected in the cells without BDM, strongly supporting the proposal that cells make use of the tension-dependent local disassembly of actin filaments by cofilin.

In accord with the above hypothesis, a tension decline in the actin stress fibers leads to the disassembly of the stress fibers, however, the possibility cannot be excluded that it is the disassembly that leads to the decline in tension in the stress fibers. To resolve this problem, simultaneous measurement of the tension decline and the disassembly of the actin stress fibers is required. We recently examined the relationship between the tension decline and stress fiber disassembly in living cells.[48] Fibronectin-conjugated beads were adhered to the endothelial cells in that study. Actin stress fibers were formed between focal adhesions underneath the bead and the focal adhesions at the cell bottom. Mechanical force was locally applied to the stress fibers by displacing the bead. The force (35 nN generated by 1 μm displacement of the bead) applied to the stress fibers and to the connected focal adhesions was estimated from the displacement of the beads embedded in the elastic substrate of the cells. When the attached bead was displaced, the force applied to the focal adhesions transiently rose, and then declined in less than a few seconds, indicating a tension decline in the stress fibers, probably

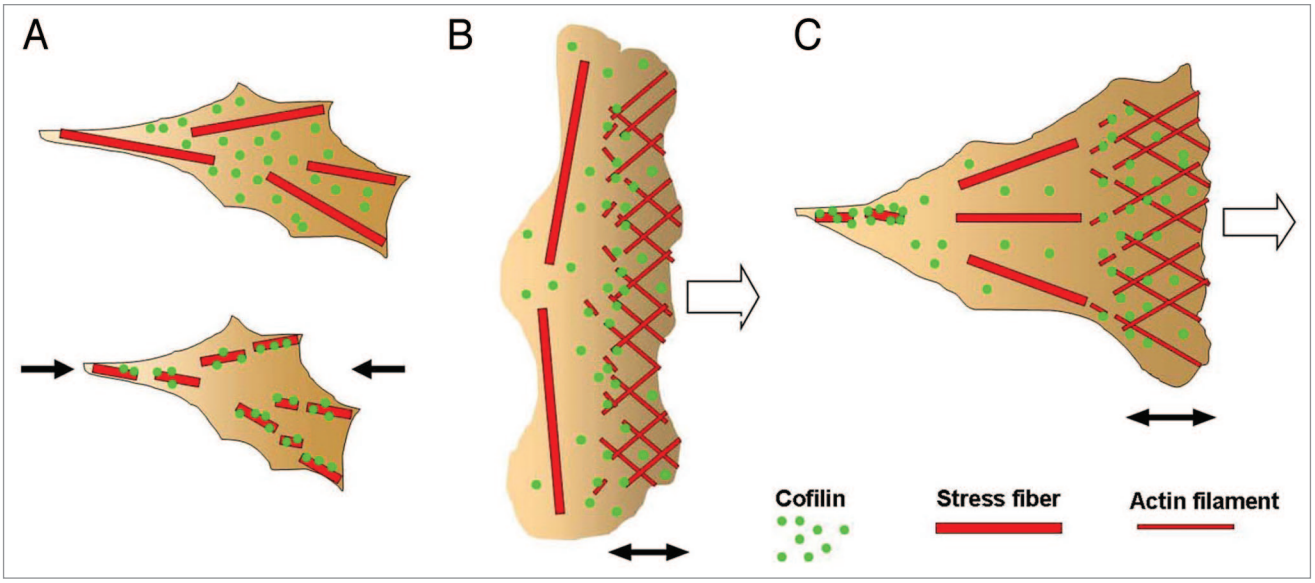

Figure 2. (A) When a certain amount of tension was generated in the stress fibers in adherent cells, the binding of cofilin to the stress fibers was reduced (upper panel). When the tension was reduced by relaxing the cell substratum (the direction is indicated by the black arrows), cofilin bound and started to disassemble the stress fibers (lower panel). **(B)** Schematic drawing of the actin cytoskeleton in a locomoting keratocyte. The actin filaments are disassembled by cofilin near the leading edge of the cell. The prominent transverse stress fibers generating a large amount of contractile force are not disassembled. **(C)** Schematic drawing of the actin cytoskeleton in an adherent cell during migration. The actin filaments are disassembled in the trailing region of the cell, where the tension in the stress fibers is low, while the stress fibers generating tension in the middle region of the cell are not disassembled. The open arrows in B and C denote the direction of cell migration. The double-headed arrows indicate the width of the lamellipodia.

due to a partial destruction of the linkage among the actin fibers within the stress fiber, which would decrease its elastic modulus. Subsequently, the same stress fibers were gradually disassembled within a period of 10 min, indicating that the decrease in tension in a particular stress fiber is followed by its disassembly, though the involvement of cofilin with this arrangement has not been examined yet.

Involvement of Tension Sensing by Actin Filaments in Migrating Cells

The potential roles of the tension-dependent local disassembly of actin stress fibers in migrating cells can be discussed in cells such as keratocyte and osteocarcinoma cells, in which the magnitude and distribution of the intrinsic contractile force generated by the actin cytoskeletons were quantitatively analyzed.[49-51] The traction forces in migrating keratocytes were estimated from the local distortion in the elastic substratum of the cells. The traction forces generated beneath the front area of a migrating keratinocyte cell are relatively small, while the forces beneath the lateral area of the cell are substantial.[49] In locomotive keratocytes, actin filaments are polymerized near the leading edge of the cells and then disassembled within a few minutes, thereby maintaining a filament length of a few μm, while, in contrast, the prominent transverse stress fibers that connect both the lateral sides of the cell are not disassembled.[50] Similar prominent stress fibers connecting the lateral sides of the cells are also found in migrating osteosarcoma cells, and are disassembled with a decline in tension by the myosin inhibitor, blebbistatin.[51] The width of lamellipodia (shown by the double-headed arrow in **Fig. 2B**) is almost proportional to the length of the actin filaments extending at the leading edge, and knockdown of cofilin by RNAi increases the width of lamellipodia,[52] suggesting that cofilin mediates the actin filament disassembly near the leading edge. These findings fit the hypothesis that the disassembly of stress fibers by cofilin is inhibited when the filaments are tensed.

The tension across vinculin, a focal adhesion protein, has been assessed in the migrating endothelial cells using a vinculin-fusion protein named "a tension sensor module"[53] that was designed based on the FRET mechanism. The FRET signal is high at disassembling or sliding focal adhesions near the trailing edge of migrating cells, implying that the tension sensed by the sensor is low (< 2.5 pN) where stress fibers are disassembled. This also agrees with the above hypothesis, and suggests that a force as small as in a pN range can presumably be sensed by actin filaments, and, when decreased, initiates the stress fiber disassembly in living cells. Proper disassembly of stress fibers is crucial for cell migration, because actin stabilization by phalloidin,[54] and enhancement of stress fiber formation[55] both inhibit cell migration. Thus, the selective disassembly of non-tensed stress fibers is crucial for cell migration; *e.g.,* cell migration is partly realized by the continual processing of actin fiber dynamics, including disassembly at the trailing edge and assembly at the leading edge of cells.

The invasion of cancer cells is enhanced by mechanical stimulation; the number of cancer cells that invaded the three dimensional matrix gel was increased when the gel was deformed by twisting small magnet beads embedded in the gel by an application of an external magnet field (i.e., mechanical stimulation to the cells in the gel). In addition, cofilin is involved in the

mechanically stimulated invasion,[56] suggesting that the mechanosensing by actin filaments plays an important role in cancer cell invasion. Elucidating the role of the tension sensing performed by actin filaments in combination with cofilin binding/unbinding will ultimately provide great insights into cell behaviors under not only physiological, but also pathophysiological conditions.

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