Chapter 28
Ion Channels Activated by Mechanical Forces in Bacterial and Eukaryotic Cells

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Abstract Since the first discovery of mechanosensitive ion channel (MSC) in non-sensory cells in 1984, a variety of MSCs has been identified both in prokaryotic and eukaryotic cells. One of the central issues concerning MSCs is to understand the molecular and biophysical mechanisms of how mechanical forces activate/open MSCs. It has been well established that prokaryotic (mostly bacterial) MSCs are activated exclusively by membrane tension. Thus the problem to be solved with prokaryotic MSCs is the mechanisms how the MSC proteins receive tensile forces from the lipid bilayer and utilize them for channel opening. On the other hand, the activation of many eukaryotic MSCs crucially depends on tension in the actin cytoskeleton. By using the actin cytoskeleton as a force sensing antenna, eukaryotic MSCs have obtained sophisticated functions such as remote force sensing and force-direction sensing, which bacterial MSCs do not have. Actin cytoskeletons also give eukaryotic MSCs an interesting and important function called "active touch sensing", by which cells can sense rigidity of their substrates. The contractile actin cytoskeleton stress fiber (SF) anchors its each end to a focal adhesion (FA) and pulls the substrate to generate substrate-rigidity-dependent stresses in the FA. It has been found that those stresses are sensed by some Ca^{2+}-permeable MSCs existing in the vicinity of FAs, thus the MSCs work as a substrate rigidity sensor that can transduce the rigidity into intracellular Ca^{2+} levels. This short review, roughly constituting of two parts, deals with molecular and biophysical mechanisms underlying the MSC activation process mostly based on our recent studies; (1) structure-function in bacterial MSCs activation at the atomic level, and (2) roles of actin cytoskeletons in the activation of eukaryotic MSCs.

Keywords Mechanosensitive channels • Membrane tension • Actin cytoskeleton • Stress fiber • Focal adhesion • Rigidity sensing

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28.1 Introduction

Mechanosensing is an indispensable function to support the life of organisms throughout the phylogeny from bacteria to human. Not only specialized mechanoreceptors, which connect to the central nervous system, like inner ear hair cells, cutaneous tactile sensors and visceral baroreceptors but also every ordinary cell can sense mechanical stimuli and utilize the information to regulate their shape, motility, differentiation and cell cycle. The known major player in cell mechanosensing is the mechanosensitive ion channel (MSC) that can be activated primarily by increased membrane tension generated by externally applied mechanical forces to cells. Among MSCs, the bacterial MSCs MscL and MscS are the best studied ones owing to their resolved 3D protein structures of the closed state. They are activated simply by increased tension in the plasma membrane (lipid bilayer) and contribute to the cell volume regulation against hypotonic challenge, thus working as a safety bulb to protect cells from osmotic rupture.

On the other hand, eukaryotic MSCs, the majority of which are Ca^{2+} permeable, seem to be activated mainly by tension in the actin cytoskeleton stress fiber (SF) that connects to the focal adhesion (FA) adhering to the cell substrate. The supramolecular complex of SF/FA/MSC has an "active-touch sensing" capability, in which actively generated contractile forces in the SF pull the substrate via an FA and activate MSCs existing in the vicinity of the FA. As stresses in FAs generated by SF dragging of substrate would depend on rigidity of the substrate, the MSCs can transduce substrate rigidity into the amount of Ca^{2+} influx across the MSCs.

MSCs might have evolved from the simple bacterial ones, which directly respond to changes in the membrane tension, to the elaborated eukaryotic ones that can actively detect mechanical properties of their immediately surrounding environments. This short article summarizes our recent studies on the differential activation mechanisms between bacterial and eukaryotic MSCs.

28.2 Activation Mechanisms in Bacterial MSCs

Bacterial MscL and MscS are the only MSCs of which 3D protein structures in the closed state are resolved at the atomic level, and MscL has been better studied due to its simpler structure. As shown in Fig. 28.1, MscL is constituted of homopentamer of a hair-pin like structured subunit having two transmembrane α-helices called inner helix (TM1, white) and outer helix (TM2, pink), displaying fivefold radial symmetry around the central pore (right panel in Fig. 28.1a) (Steinbacher et al. 2007). Neighboring TM1s cross and interact each other near the cytoplasmic side through hydrophobic interactions, forming the most constricted pentagon-shaped hydrophobic part (called "gate") of the pore. The crossings stabilize the closed state of MscL. The issue is to understand the underlying molecular mechanism during the course of channel opening (gating) driven by tension increase in the membrane;

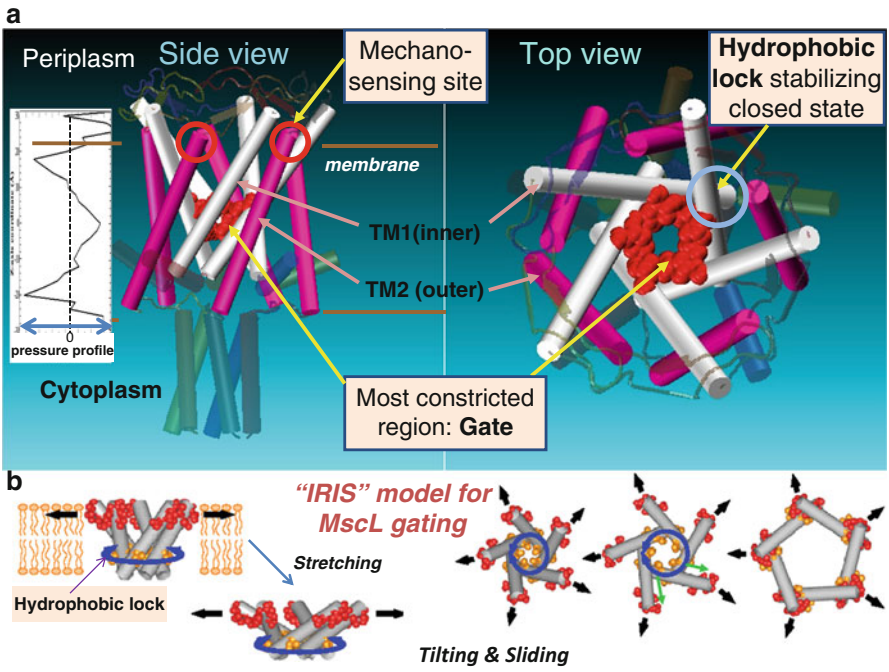

Fig. 28.1 3D structure and a gating model of *E. coli* MscL. (**a**) Schematized 3D structure of MscL based on a latest study (Steinbacher et al. 2007). Some hypothetical functional sites and transmembrane pressure profile (most *left*) are indicated. (**b**) IRIS model for MscL gating, where membrane stretch induces tilting of helices (*left*) and radially outward sliding of the crossings between inner TM1 helices (*right*) (Modified from (Sawada et al. 2012))

which amino acid in the channel protein senses tensile forces and how the sensed forces lead to the channel/gate opening of MscL.

We identified potential tension-sensing site in the MscL protein by using single channel current recordings in combination with site-directed mutagenesis. Amino acid (AA) residues facing the lipid bilayer were substituted one by one with a hydrophilic AA (asparagine, N) in order to weaken the hydrophobic interaction between the AAs and lipids to search which AA(s) is crucial in sensing tension in the membrane. The asparagine substitution of one of seven residues at the periplasmic end of transmembrane helices caused loss of MscL mechanosensitivity, whereas the substitution of the residues at the core of the bilayer did not affect the channel gating (Yoshimura et al. 2004; Yoshimura and Sokabe 2010). This result indicates that the hydrophobic interaction between lipids and one or some of the residues near the periplasmic surface may act as a tension sensor of MscL. This is consistent with the transmembrane tension (pressure) distribution with two peaks near the inner and outer membrane surfaces (most left graph in Fig. 28.1a).

Based on the above implication, we have proposed a model for MscL gating called IRIS model (Yoshimura et al. 2004). Upon membrane stretch, the AA

residues near the outer surface of the membrane are dragged by lipids and TM1/TM2 helices are gradually tilted (left panel in Fig. 28.1b) accompanied by outwardly radial sliding of the crossings between TM1 helices, leading to gate expansion (right panel in Fig. 28.1b). This model has been supported by our recent single molecular imaging of closed and open structures of MscL by cryo-electron microscopy (Yoshimura et al. 2008).

To get insight into more detailed features of structural dynamics of MscL opening at the atomic detail, molecular dynamics (MD) simulations were performed (Sawada et al. 2012). We constructed a molecular model using the closed structure of MscL (Steinbacher et al. 2007) embedded in a lipid bilayer and immersed in water molecules. MD simulations were conducted under an increased membrane tension that was generated by reducing the lateral pressure only in the bilayer. Simulation time of all-atom MD of proteins is in general limited for a few tenths of nanoseconds (ns) to obtain reliable results, which is apparently much shorter than that (> a few hundred microseconds) required for actual MscL opening. Therefore, we focused on the two mechanisms critical for initiating MscL opening; (1) which residue(s) has the most potent and stable interaction with the surrounding lipids (identification of tension sensor) and (2) how the received force by the sensor induces expansion of the gate of MscL. Results principally succeeded in reproducing the initial process predicted by the "IRIS" model (Fig. 28.1b), that is, tilting of TM1/TM2 helices accompanied by sliding of the TM1 crossings towards channel opening.

To determine the major tension-sensing sites (tension sensor) in MscL, we estimated the interaction energy between individual AA residues from Gly76 to Ala89 in TM2 helix and surrounding lipids. Surprisingly, the interaction energy between Phe78 and lipids is conspicuously much lower than that of any other residues. Since the tension sensitivity at the level of individual AA residues depends on the mechanical strength of AA-lipids interaction, Phe78 was thought to act as the major tension sensor of MscL. Actually, TM2 was dragged by lipids specifically at Phe78 and tilted (Fig. 28.2a). Interestingly, aromatic ring of Phe78 was frequently facing the CH_2 residues of lipid acyl chains, implying that CH/Π interaction (Tsuzuki et al. 2000; Shanthi et al. 2010), as depicted in Fig. 28.2b, is the physicochemical mechanism for conspicuously strong interaction between Phe78 and lipids, by which Phe78 can act as the major tension sensor of MscL.

Next, in order to know the details how the gate is expanded, we sought AAs that stabilize the closed gate at the crossings between neighboring TM1 helices (see Fig. 28.1a), and found that the residues (Val16, Leu19 and Ala20) on a TM1 helix come into contact with Gly22 and Gly26 on the neighboring TM1 helix (left panel in Fig. 28.2c). Upon membrane stretching, Gly22 began to come into contact with its neighboring TM1 helix in ∼1 ns, fitting into a pocket formed by Val16, Leu19 and Ala20, then Gly26 instead of Gly22 began to come into contact with the pocket (middle to right panels in Fig. 28.2c). Time profile of interaction energies at those interacting AAs between neighboring TM1 helices revealed that an energy barrier exists during the 2 ns simulation. The potential energy difference between the two states separated by the barrier was calculated to be ca. 25 kcal mol^{-1} (42 k_BT), which value is comparable to the experimentally estimated free energy

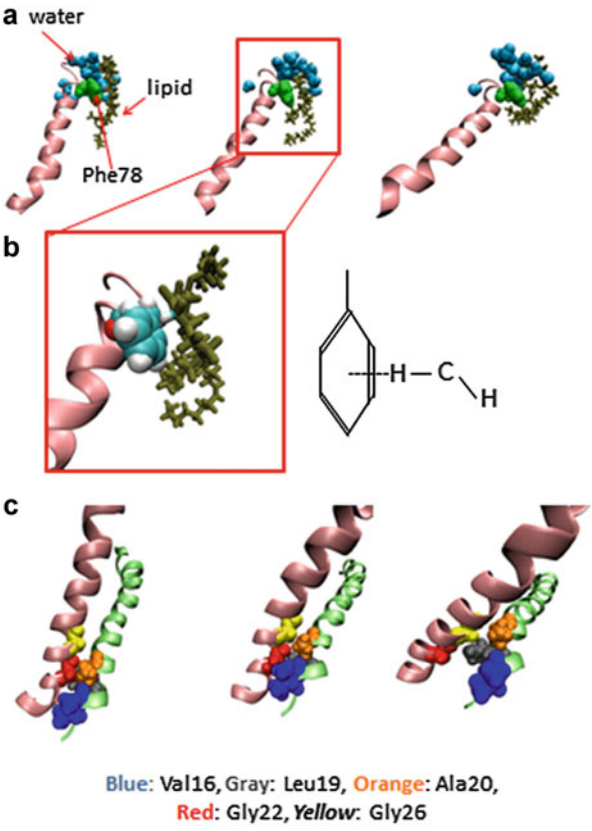

Fig. 28.2 Detailed structural changes at the putative tension sensor and gate at the atomic level, during the initial phase towards channel opening. (**a**) Snapshots of lipid dragging of Phe78 on a TM2 at 0, 2, 4 ns after membrane stretch, which causes TM2 (and TM1) tilting. (**b**) Interaction between aromatic ring of Phe78 and CH of a lipid acyl chain (CH/π interaction). (**c**) Snapshots of sliding between neighboring TM1s at 0, 3, 4 ns after stretch onset (Modified from (Sawada et al. 2012))

difference (∼38 k_BT) between the closed and the first sub-conducting state of MscL (Sukharev et al. 1999). It is rather surprising that such a limited MD calculation gives a good number, the model might reflect an essence of initial process of MscL gating. Detailed calculations including other interactions that may contribute to the MscL gating are in progress.

28.3 Activation Mechanisms in Bacterial MscS

Another bacterial MS channel MscS has a smaller conductance (1 nS), a lower threshold for activation and a much more complicated structure in comparison with those of MscL. MscS is a homoheptamer of a subunit having three transmembrane α-helices called TM1, TM2, and TM3 (middle panel in Fig. 28.3a), displaying sevenfold radial symmetry around the central pore (right panel in Fig. 28.3a) with a large cage-shaped cytoplasmic domain having side windows (Bass et al. 2002) that are implicated to work as a molecular sieve against large sized molecules (left

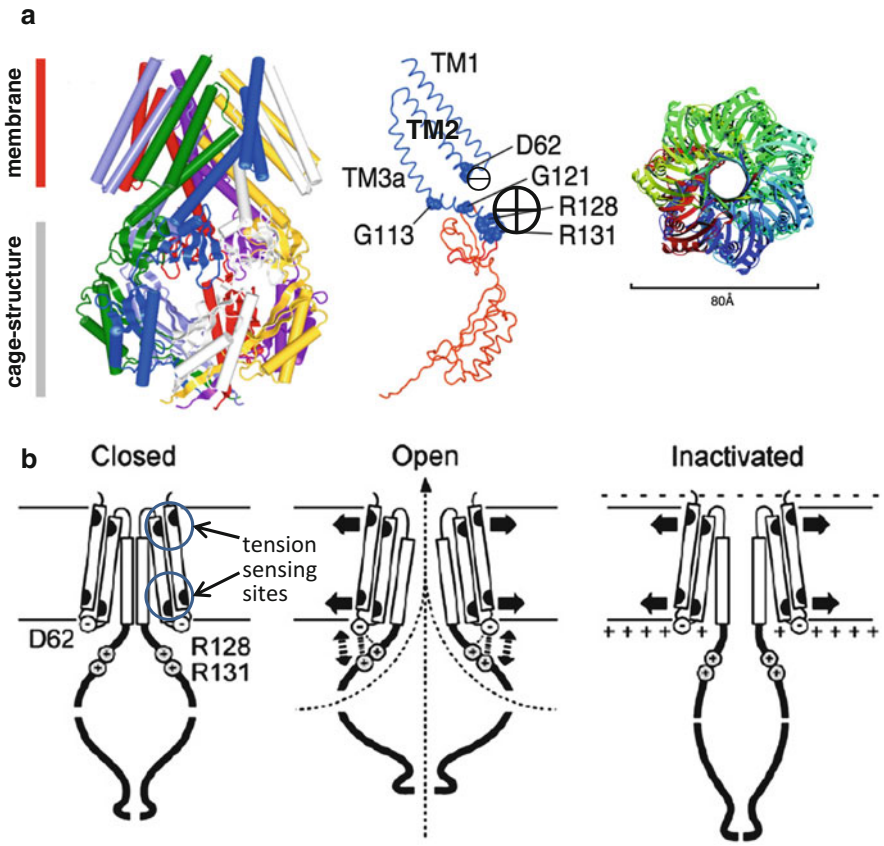

Fig. 28.3 3D structure of MscS and its gating model. (**a**) Side view of the whole (*left*) and subunit (*middle*) structures, and a top view of the whole structure, see text for details (Modified from (Bass et al. 2002)). (**b**) A model for the MscS activation (*opening*) and inactivation. Asp62 is located in the loop that connects TM1 and TM2. Arg128 and Arg131 are located in the upper surface of the cytoplasmic vestibule (*left panel*). When the channel opens, Asp62 forms a salt bridge with Arg128 and Arg131 (*middle panel*). When the cytoplasmic potential is positive, the electrostatic interaction is dissociated by the positive surface potential (*right panel*). The resulting shrinkage of the cytoplasmic vestibule brings about channel inactivation (Modified from (Nomura et al. 2008))

panel in Fig. 28.3a, b). TM3s line the pore and some AA residues of TM3 near the cytoplasmic surface of the membrane are postulated to form the main gate of MscS.

MscS as well as MscL can be activated exclusively by tension in the membrane. In contrast to the conformational changes during the MscL opening (tilting and sliding), MscS seems to open through a lateral expansion of TM helices along the membrane plain (Fig. 28.3b). How does membrane tension cause such a conformational change in MscS? As did in MscL, we tried toidentify the tension

sensing site of MscS using the asparagine scanning method, and found that two separate tension sensing sites are located near both surfaces of the membrane (left panel in Fig. 28.3b) (Nomura et al. 2006), which conforms the lateral expansion model of TMs during MscS opening. This configuration of tension sensing sites may also partially account for the higher mechanosensitivity of MscS than MscL, since the number of mechanosensing sites of MscS can be estimated at 14 (2 sites/subunit $\times$ 7 subunits) a much larger number than that (five) of MscL. The sensed forces at the tension sensing sites may be transmitted through linkers between helices and/or lateral interactions among helices, eventually lead to a lateral expansion of TM3 helices. However, in the case of MscS opening, things are not so simple.

The large cytoplasmic domain has a crucial role in the MscL opening. As depicted in Fig. 28.3b, there is an electrostatic interaction between negatively charged Asp62 at the TM1-TM2 linker and positively charged Arg128/131 at the cytoplasmic domain. We found that destruction of this ionic interaction by site-directed mutagenesis caused loss of function (LOF) mutants (Nomura et al. 2008). Presumably, lateral expansion of TM1 and TM2 pulls up and distends the cytoplasmic domain (middle panel in Fig. 28.3b) (Machiyama et al. 2009), followed by stretching out of the linker directly connecting to the gate of TM1 to open the channel. Interestingly, under a large and/or long lasting hypotonic stress MscS transits to an inactivated state (right panel in Fig. 28.3b), where the channel never opens even with a very strong mechanical stimulus (membrane stretch). MscS can recover its closed state only when the membrane tension returns to the resting level. Inactivation of MscS can also be induced by membrane depolarization, which would occur when MscS opens for a long time due to a long exposure to hypotonic shock, because preferred anion permeability of MscS will cause anion out-flux from cells. This is a very smart way to prohibit excess loss of valuable cytoplasmic components. Another interesting potential function of the cytoplasmic domain is that it may work as a cytoplasmic osmometer. When MscS opens under hypotonic shock, cytoplasmic solutes and water will flow out through MscS, but large sized molecules that cannot permeate the side windows (14 Å in diameter) of the cytoplasmic domain will be condensed around the outside surface of the cage-domain, leading to an increased osmolarity there. This induces water outflow from the cage to the cytoplasm, leading to a shrinkage of the cage (right panel in Fig. 28.3b), which would uncouple the electrostatic interaction between TM3 and the cage to induce inactivation of MscS. This may be another protection mechanism to inhibit excess loss of cytoplasmic components. Based on the hypothetical model proposed here, inactivation as well as activation of MscS largely depends on the interaction of TM2 with TM3 and TM1. In other words, TM2 may work as a modulator to regulate the threshold for activation and inactivation of MscS.

While MscL has a large conductance (3 nS), a high activation threshold and a simple regulatory system, MscS has a smaller conductance (1 nS), a lower threshold, and a subtle regulatory system, by which they may play differential roles as an emergent device and a housekeeping one, respectively.

28.4 Activation Mechanisms in Eukaryotic MSCs

It has been proposed that eukaryotic MSCs are activated mainly by tension in the cytoskeleton based on pieces of indirect evidence (Sokabe et al. 1991; Sokabe and Sachs 1992). To test this hypothesis, we employed cultured human umbilical vein endothelial cells (HUVECs). HUVECs carry a 35 pS Ca^{2+} permeable MSC (Naruse and Sokabe 1993), which enabled us to monitor the channel activation through the measurement of intracellular Ca^{2+} increases as well as whole cell currents across the MSC. In addition, HUVECs have well developed F-actin based stress fibers (SFs) that anchor to FAs (Fig. 28.4 left). We hypothesized that SFs act as an intracellular force transmitter to activate the MSCs located in the vicinity of the integrins in the FA to which SFs are anchored (Fig. 28.4, right).

To verify this hypothesis we developed a sophisticated method by which we can apply localized mechanical stimuli onto FAs via SFs while monitoring Ca^{2+} influx in and near FAs by high speed near field microscopy (Hayakawa et al. 2008). Aseptically prepared HUVECs were plated on a fibronectin (FN) coated cover slip and a glass bead (5–10 μm) coated with FN was attached on the apical surface of a HUVEC. Focal proteins (such as integrin and vinculin) and F-actins were assembled in the membrane region attaching the bead to form FAs beneath the bead within 15 min. Each FA was connected via an SF to a preformed FA at the basal cell surface. A short pulse of stretch was applied through this structure to the basal FAs by displacing the bead with a piezo-driven glass pipette, while monitoring Ca^{2+} transient near the basal cell surface using the Ca^{2+} indicator fluo-3.

Immediately after a stimulation (0.1–2 μM displacement of the bead) an intracellular Ca^{2+} increase was observed around basal FAs, which was strongly

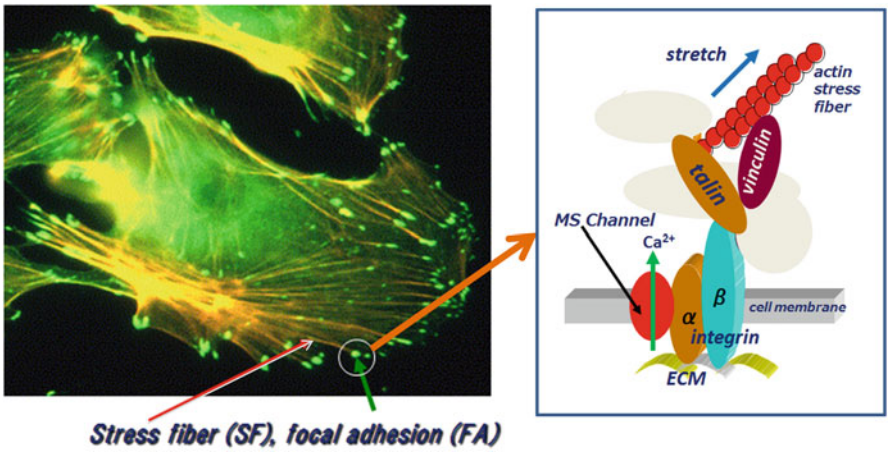

Fig. 28.4 Fluorescent image of HUVECs. Strand like SFs terminate at spot shaped FAs. *Right panel* shows hypothetical SF/FA/MSC complex, in which MSC is activated by force (tension) transmitted through SF and FA (integrin)

inhibited by external Ca^{2+} deprivation or by the potent MSC blocker gadolinium (Gd^{3+}), whereas thapsigalgin, which depletes intracellular Ca^{2+} stores, did not show any significant effect. As the F-actin depolymerizing drug cytochalasin D also inhibited the Ca^{2+} increase, it is highly likely that the stretch induced Ca^{2+} transient was mediated by the MSCs that are activated by the force transmitted along SFs.

To directly test the above idea, phalloidin coated fluorescent plastic beads, which selectively bind to actin filaments and SFs, were microinjected into the cell through a glass pipette for whole cell current recordings. A traction force was applied to a bead aggregate attached on an SF using the laser trapping technique while recording the resulting whole cell current and Ca^{2+} increase (Fig. 28.5a). An inward current and a Ca^{2+} increase were detected immediately after a force application to the bead aggregate, strongly supporting our hypothesis that tension in the SF activates MSCs. It was found that stretching a single SF could activate 20–40 MSCs and that force required to activate a single MSC was as small as 1 pN (Hayakawa et al. 2008).

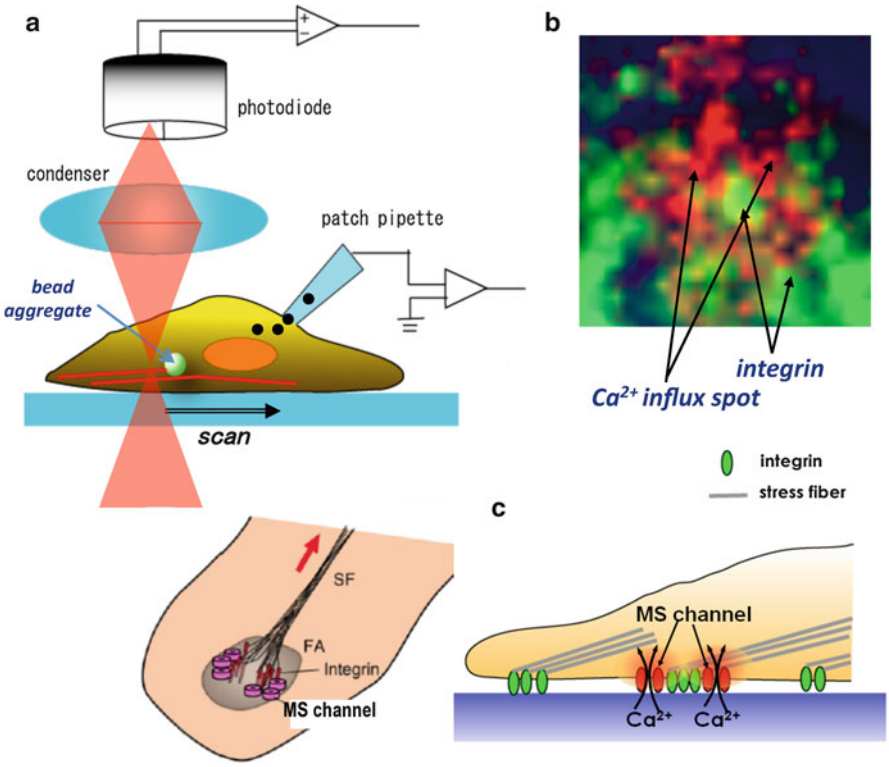

Fig. 28.5 Simultaneous measurements of whole cell currents and Ca^{2+} influx spots in response to an SF stretch. (**a**) Experimental setup. (**b**) A live fluorescent image of integrins and Ca^{2+} influx spots (Modified from (Hayakawa et al. 2008)). (**c**) Schematic diagram of hypothetical SF/FA/MSC complex and SF/integrin/MSC complex

We also could visualize, by ultra-fast near field microscopy, individual Ca^{2+}-influx spots that occurred near FAs within a few msec after a stretch. The Ca^{2+}-influx spots had a size as small as 300 nm and quantitative estimation suggested that the smallest spots reflect the images of the moment of Ca^{2+}-influx through single MSCs (Fig. 28.5b). Analysis of the spatial distribution of the Ca^{2+}-influx spots and integrins showed that MSCs are located as close as a few hundred nm apart from FAs (integrins). A hypothetical model for mechanosesning machinery composed of SF, FA and MSC is shown in Fig. 28.5c. The mechanism how the conveyed force via SF/integrins transmits (e.g., via membrane or submembraneous cytoskeletons) to MSCs awaits future studies. It should be noted that our results do not deny the role of membranes as a force transmitter but rather show up the actin stress fiber as an accessory system that gives new functions to MSCs, such as force concentration, remote force sensing, and force direction sensing. Unfortunately, as the molecular entity of the MSC in HUVECs has not been identified yet, we cannot discuss on the detailed supramolecular architecture of the SF/FA/MSC complex.

28.5 Active Mechanosensing: Rigidity Detection

Cellular motility, spreading, proliferation and differentiation are critically influenced by the substrate rigidity. To sense substrate rigidity, cells apply traction forces to cell–substrate adhesions via SFs and measure mechanical responses of the substrate. The supra-molecular complex of SF/FA/MSC shown in Fig. 28.5c may represent an ideal device to execute this task. In contrast to the Ca^{2+} transient induced by external forces, substrate rigidity sensing is induced by intrinsic mechanical stimuli, such as imposing traction forces generated in contractile SFs onto the ECM via SF/FA complex. MSCs located in or near FAs would convert the substrate-rigidity dependent stress generated in the FA into the level of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) via a local activation of the MSCs.

If this hypothesis stands and cells intermittently detect substrate rigidity, it is expected that cells will show spontaneous Ca^{2+} oscillations under no extrinsic mechanical stimulation. As the generated stress in FAs would larger in the cells on a rigid substrate than that on a soft substrate, the peak amplitude of the Ca^{2+} oscillation should depend on the substrate rigidity (Fig. 28.6a). Actually, HUVECs cultured on stiffer substrates showed spontaneous Ca^{2+} oscillations with larger amplitudes than those on soft substrates (top and bottom panels, respectively, in Fig. 28.6b) (Kobayashi and Sokabe 2010).

It has been implicated that rigidity sensing by cells is used for controlling cell migration. As can be seen in Fig. 28.6b (middle panel), the amplitudes of Ca^{2+} oscillations are much larger in the cells located at the boundary between soft and stiff substrates during durotaxis (mechano-taxis), strongly suggesting that spontaneous Ca^{2+} oscillations are used to search substrate with a stiffness proper for the cells during their migration. The rigidity sensing machinery SF/FA/MSC seems to be used more lively in the process of cell migration. Moving cells have

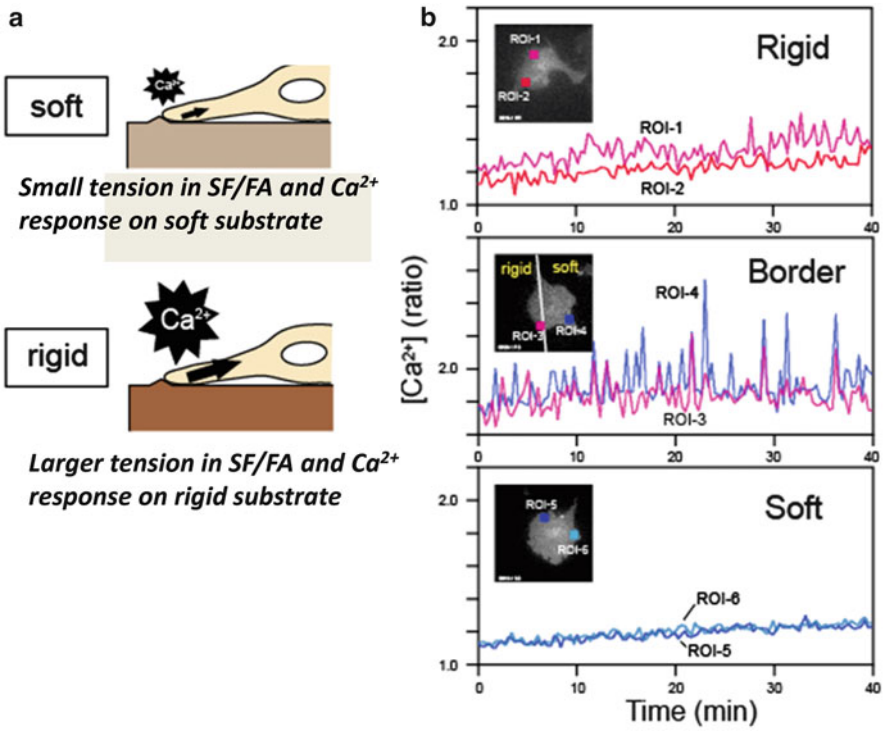

Fig. 28.6 Rigidity sensing by SF/FA/MSC complex. (**a**) Actively generated contractile forces in SF pull the substrate: tension in the SF/FA complex may depend on the substrate rigidity. (**b**) Spontaneous Ca^{2+} oscillations on a rigid (*upper*) and a soft (*lower*) substrate. Rigidity sensing is much more vigorous on the border between rigid and soft substrates during durotaxis (*middle*) (Modified from (Kobayashi and Sokabe 2010))

a rear-to-front calcium gradient with the lowest concentration at the front, and transient increases in the $[Ca^{2+}]_{cyt}$ have been thought to facilitate retraction at the rear by increasing SF contractility, or promoting FA disassembly (Ridley et al. 2003; Kiyoshima et al. 2011). Recently it has been reported that local increases in the $[Ca^{2+}]_{cyt}$ via TRP melastatin 7 (TRPM7) are required for cell migration or chemotaxis (Wei et al. 2009). As TRPM7 is a Ca^{2+}-permeable MSCs (Numata et al. 2007), it is critically important to examine the involvement of TRPM7 in sensing substrate rigidity by cells.

28.6 Summary and Perspectives

Bacterial MscL is activated exclusively by membrane tension and used to sense changes in the global tension in the cell membrane, followed by proper outflow of cytoplasmic components in order to adjust the intracellular osmolarity to the

extracellular one. This function seems to be sufficient for MscL to work as an emergent safety valve protecting cells from lysis under a severe hypotonic downshock and the underlying activation mechanism in MscL is relatively simple. By contrast, MscS has a more sensitive and elaborated mechano-gating mechanism to deal with subtle environmental changes, working as a house-keeping device. Particularly, the inactivation mechanism of MscS, which is probably used to protect cells from losing excess amount of valuable cytoplasmic components, works in an ingenious manner. Further analyses of the gating mechanisms in bacterial MSCs would give the first complete showpiece, in terms of structure-function at the atomic level, among ion channels. Notwithstanding, MscS has not been inherited by eukaryotic cells; only limited species seem to inherit MscS homologues (Nakayama et al. 2007; Haswell et al. 2008). This may be because most eukaryotes have succeeded in developing various systems to protect the cells vulnerable to hypotonic challenge from direct exposure to such a dangerous stimulus.

Eukaryotes have evolved MSCs in several other directions. First, they utilize MSCs as a mechanosensor for specialized mechanoreceptors, such as inner ear hair cells, skin mechanoreceptors, visceral baroreceptors and muscle mechanoreceptors, to control their body reactions. Second, eukaryotic MSCs have been employed as a mechanosensor for nonsensory cells to detect mechanical forces generated by flows of body fluid, movements of organs and skeletal muscles, and gravity, making use of those mechanical cues for local (without nervous system) regulations of tissues and organs, including blood vessels, intestines, and bones. Third, eukyariotic MSCs are working as a mechanosensor to detect stresses created by intrinsic forces produced in cells, most of which are produced by polymelization-depolymerization dynamics of microtubules and microfilaments or contraction of actomyosin fibers like SFs. This function is critically important in the regulation of fundamental cell functions, such as growth, proliferation, differentiation and migration of cells. Finally, as a variation of the third type of cell mechanosensing, eukaryotic cells have evolved active touch sensing mechanisms, by which cells can detect mechanical properties (e.g., stiffness and viscosity) of their surrounding environments, including neighboring cells and substrates. This is realized by concerted operations among MSCs, contractile actin cytoskeletons and adhesive molecules. The active mechanosesning is used for cell migration towards the substrate with a proper rigidity, which may play crucial roles in embryonic development, wound healing, tissue regeneration and cancer invasion.

One of the characteristic features across eukaryotic MSCs is their association with a variety of accessory proteins such as cytoskeletons and adhesive molecules. This gives the MSCs, beyond the simple membrane tension sensing in bacterial MSCs, additional functions such as remote force sensing (via cytoskeletal linkage between membrane proteins and the nucleus), force direction sensing (e.g., tip-links between stereo-cilia in hair cells) and active mechanosensing as described above. One of the most important problems in the coming decade is to resolve the structure-function of supramolecular complex of MSC/accessory proteins, as well as molecular identification of eukaryotic MSCs. Moreover, identification and characterization

of MSC-specific downstream signaling cascades remains challenging subjects to understand and to establish the biological and physiological roles of MSCs.

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