CHAPTER 3

Activation of Mechanosensitive Ion Channels by Forces Transmitted Through Integrins and the Cytoskeleton

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I. OVERVIEW

Mechanosensitive (MS) ion channels play a central role in the process of cellular mechanotransduction by which living cells convert mechanical signals into chemical and electrical responses. Current views of the mechanism of MS channel gating focus almost entirely on local modulation by plasma membrane tension or by ''gating springs'' within the underlying submembranous cytoskeleton (CSK). However, cells within many solid tissues commonly experience mechanical stresses that are transmitted over extracellular matrix (ECM) scaffolds to specific transmembrane integrin receptors. Integrins physically anchor the ECM to both the submembranous CSK and the deeper CSK (i.e., the microfilament–microtubule–intermediate filament lattice), and thus, they also constantly experience forces that are generated

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within cytoskeletal contractile actomyosin filaments and exerted on these same adhesion sites. We have previously proposed that cells use tensegrity architecture to mechanically stabilize their shape by maintaining prestress in the interconnected ECM–integrin–CSK lattice and that the activity of certain MS channels may be modulated through tension transfer *inside the cell* via this tensed structural network, rather than through direct lipid bilayer distortion. Here, we review this tensegrity-based mechanism for MS channel regulation in light of recent work which confirms that integrins provide a specific path for stress-dependent activation of MS channels. We also discuss potential molecular mechanisms that might mediate this tensegrity-based mechanotransduction mechanism for both short- and long-range force transfer through living cells.

II. INTRODUCTION

Cellular mechanotransduction—the process by which cells convert mechanical signals into changes in intracellular biochemistry—plays a central role in tissue development, as well as in many disease processes (Ingber, 2003a, 2006). Cells within all living tissues encounter mechanical forces continuously within a changing dynamic environment, and they have evolved an exquisite mechanosensory system to perceive and respond to these forces (Ingber, 1997b, 2006).

Cells first sense mechanical stresses, like other external signals, when they impinge on the cell surface. Early work on the mechanism of mechanosensation revealed that virtually all cell types express MS ion channels on their plasma membranes that alter their activity (i.e., either become activated or deactivated) when mechanically stressed (Hamill and McBride, 1997; Sukharev and Corey, 2004). In fact, the earliest recorded response to force application is a change in electrical activity which results from opening of MS channels and occurs within milliseconds (Sachs, 1992). Due to their fast response, MS channels mediate specialized sensory functions such as hearing, touch, and vestibular function (Hamill and Martinac, 2001; Sukharev and Anishkin, 2004; Sukharev and Corey, 2004). However, MS channels are also present in nonsensory cells such as endothelium, smooth muscle, and heart cells. In these parenchymal cells, MS channels regulate a number of biochemical and physiological responses, including strain-induced endothelial cell orientation, activation of protein kinases, and secretion of inflammatory mediators (Naruse *et al.*, 1998a,b,c).

Initially, it was assumed that forces applied to the cell surface activate MS channels as a result of localized distortion of the plasma membrane which results in increased tension in the lipid bilayer that is transmitted directly to

the channel molecule (Fig. 1A) (Martinac *et al*., 1990; Hamill and McBride, 1993, 1994; Sukharev *et al*., 1994; Hase *et al*., 1995; Hamill and Martinac, 2001; Kloda and Martinac, 2001a,b,c; Martinac, 2001; Perozo and Rees, 2003). This appears to be true for certain MS channels; however, many others appear to require interactions with elements of the submembranous CSK for their activation or regulation (Corey and Hudspeth, 1983; Guharay and Sachs, 1984; Hamill and McBride, 1996, 1997; Gillespie and Walker, 2001; Cho *et al*., 2002). In general, this is still thought to be a local phenomenon that results from membrane distortion-dependent alteration of the underlying submembranous CSK that then indirectly triggers MS channel activity via internal CSK-associated "gating domains" (Fig. 1B).

Meanwhile, separate studies on how cells sense and respond to mechanical stresses transmitted through ECM have revealed that these forces are preferentially transferred into cells via transmembrane integrin receptors

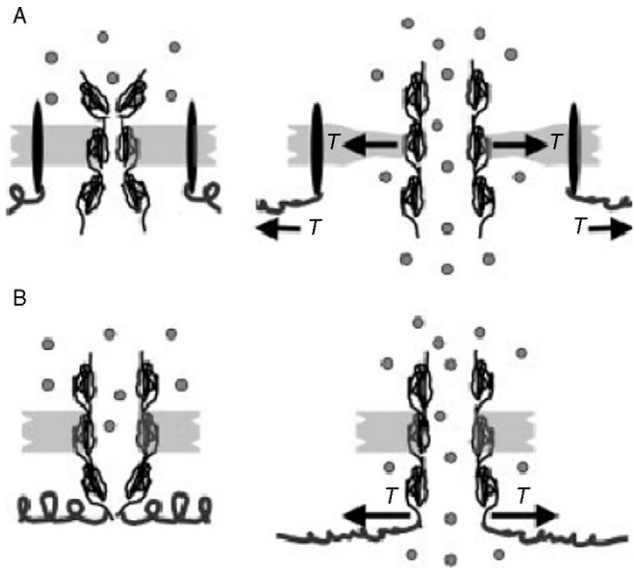

FIGURE 1 The bilayer (A) and tethered (B) models that have been proposed to explain the mechanosensitivity of MS channel gating. (A) Diagrammatic representation of an MS ion channel that alters its conformation and changes its opening and closing rates when the membrane bilayer distorts, thereby exerting tensional forces (*T*; arrow indicates direction) directly on the channel molecule. Lipid bilayer distortion may be produced by surface shear forces or potentially by cytoskeletal distortion of transmembrane integral membrane molecules that tightly associate with the lipid bilayer (black ovals). (B) In the tethered model, an MS ion channel experiences tensional forces that are transmitted directly from the internal CSK. These forces stimulate ion flux by tugging on the cytoplasmic portion of the channel that acts as a "gating spring" and opens the pore when tensed. Adapted from Ingber (2006).

that cluster together within specialized multimolecular anchoring complexes, known as focal adhesions (Wang *et al.*, 1993). Integrins and tightly associated focal adhesion molecules, such as talin, vinculin, and paxillin, also mediate outward transfer of cell tensional forces from the contractile actin CSK to external ECM adhesions on the outside surface of the cell (Geiger *et al.*, 2001). Thus, integrins are now viewed as bidirectional mechanoreceptors; however, they are not signaling receptors themselves, and thus they must partner with other transduction molecules within the focal adhesion to mediate mechanochemical conversion (Ingber, 1991).

Integrins mechanically couple the ECM to the deep internal CSK (microfilament–microtubule–intermediate filament lattice) and interconnected nuclear scaffolds by forming a macromolecular complex with underlying focal adhesion proteins. Because integrins are anchored to ECM that is relatively rigid compared to the cell, they are able to resist cell-generated traction forces and thereby maintain the cell in a state of isometric tension (i.e., tensionally "prestress" the cell and CSK) (Ingber, 1997b, 2003b, 2006; Stamenovic and Coughlin, 1999; Wang *et al.*, 2001). On the basis of these and other observations, we previously proposed that living cells are organized as "tensegrity" structures that gain their shape stability by maintaining tensional prestress within an interlinked network of opposing tension and compression elements (Fig. 2A) (Ingber and Jamieson, 1985; Ingber, 1993, 2003b). In this type of structural system, stresses applied locally to key surface anchoring molecules result in force focusing and channeling (potentially over long distances) through the discrete network linkages that connect the elements that comprise the structure. In addition, network shape stability and the efficiency of force transfer through the lattice may be modulated by altering the level of tensional prestress in the system. Hierarchical tensegrities built from multiple tensegrity modules connected by similar rules also can be constructed; these exhibit coordinated behavior between part and whole similar to that observed in all living structures (Ingber, 1997b, 2003b).

Importantly, experimental work has confirmed that many different types of cells use tensegrity to stabilize their shape (Wang *et al.*, 1993; Ingber, 1997b, 2003b, 2006; Komulainen *et al.*, 1998; Ralphs *et al.*, 2002; Brangwynne *et al.*, 2006; Kumar *et al.*, 2006), and that forces transmitted over integrins are preferentially channeled throughout cytoskeletal filaments in the cytoplasm, resulting in stress concentrations at distant sites (e.g., on the nucleus and membrane at the opposite pole of the cell) (Maniotis *et al.*, 1997; Wang *et al.*, 2001; Hu *et al.*, 2003, 2004, 2005). The dependence of cell, tissue and organ mechanics, as well as many biological responses, on cytoskeletal prestress also has been confirmed experimentally (Ingber, 2003b, 2006).

Most importantly in the present context, the tensegrity model suggests that certain MS channels might be activated by external forces that are

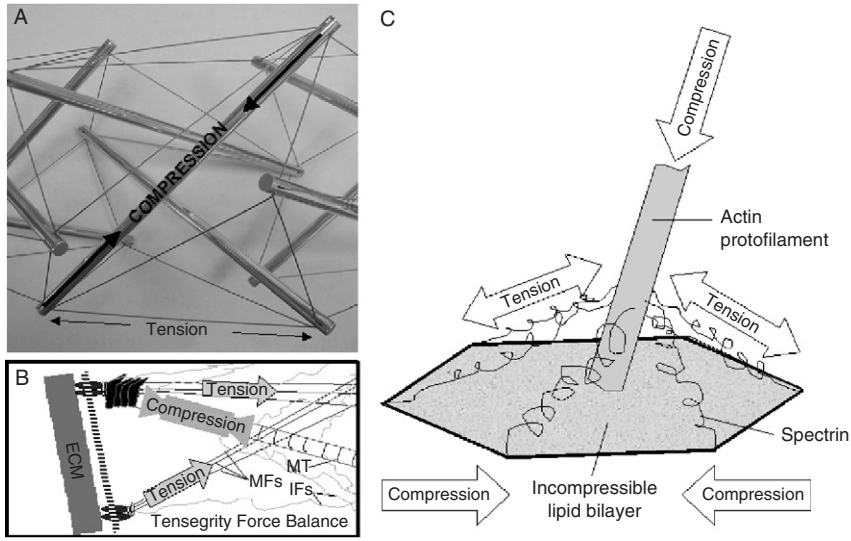

FIGURE 2 Tensegrity architecture and its use by living cells at different size scales in the CSK. (A) A photograph of a tensegrity structure with isolated compression struts (aluminum bars) and tension elements (metal wires) labeled to visualize the tensegrity force balance based on local compression and continuous tension which prestresses (and thereby mechanically stabilizes) the entire structural network. (B) A schematic diagram of the complementary force balance between microfilaments (MFs) and intermediate filaments (IFs) that transmit tension to compressed microtubules (MTs) and relatively noncompressible regions of the underlying ECM that balance these forces, and thereby establish a tensegrity force balance in the whole CSK (the submembranous CSK is not shown in this view). A, B (Ingber, 2003b), reproduced with permission from "The Company of Biologists." (C) A tensegrity force balance is established in the submembranous CSK at a smaller size scale as a result of relatively rigid actin protofilaments and noncompressible regions of the lipid bilayer resisting tensional distortion of flexible spectrin cables. Based on work of Sung and Vera (2003).

transmitted across integrins and associated cytoskeletal molecules within focal adhesions or the deeper CSK, rather than through direct lipid bilayer distortion alone (Ingber, 1997b). In this chapter, we describe this potential mechanism for integrin-dependent activation of MS channels in greater detail, discuss potential molecular mediators of this response, and review recent advances in this area.

III. CONVENTIONAL VIEWS OF MS CHANNEL GATING

MS channels range from simple two transmembrane-spanning domain-containing proteins in bacteria to large multiprotein complexes in higher organisms (Hamill and Martinac, 2001). The MS channels that have been

identified and cloned in prokaryotes and eukaryotes are divided into different families, including DEG/ENaC (degenerins/epithelial sodium), TRP (transient receptor potential), K^+_{Ca}, Kir (inward rectifier potassium), two pore K^+, MscS (small conductance MS), MscL (large conductance MS), and archeal MS channels (Hamill and Martinac, 2001; Sukharev and Corey, 2004).

Two potential mechanisms have been suggested to explain the mechanosensitivity of MS channel gating: the bilayer model and the tethered model (Hamill and McBride, 1997; Hamill and Martinac, 2001; Martinac, 2004). In the bilayer model, local tension in the lipid bilayer of the cell's surface membrane is alone sufficient to alter MG channel activity directly (Fig. 1A) (Martinac *et al.*, 1990). This possibility is supported by the finding that certain prokaryotic MS channels, such as MscL and MscS, retain their mechanosensitivity when they are purified and reconstituted into pure lipid vesicles (Martinac *et al.*, 1990; Sukharev *et al.*, 1993, 1994; Opsahl and Webb, 1994; Hase *et al.*, 1995; Hamill and McBride, 1997; Kloda and Martinac, 2001a,b,c; Martinac, 2001; Perozo and Rees, 2003), although whether this mechanism is used in living cells still remains unclear.

In contrast, the tethered model suggests that a portion of the MS channel functions like a "gate" that is tethered to molecular elements in the CSK inside the cell (or to components of the ECM outside the cell) which actually sense the mechanical stress and transmit it to the channel. Hence, these connecting proteins act like gating springs that change MS channel gate opening and closing kinetics when mechanically stressed (Guharay and Sachs, 1984; Howard *et al.*, 1988; Hudspeth and Gillespie, 1994; Huang *et al.*, 1995; Hamill and McBride, 1996). In this gated model, local stress-induced displacement of the channel with respect to the CSK, such as might occur from generalized distortion of the membrane relative to its underlying submembranous CSK (or overlying ECM) due to fluid shear, would cause channel gating. Thus, the mechanosensitivity of a membrane channel protein might be affected by local mechanical properties of the adjacent lipid bilayer, submembranous CSK, or adjacent ECM.

The submembranous CSK is a specialized part of the cortical CSK composed of an actin–spectrin–ankyrin network that structurally supports the fluid bilayer and provides the cell membrane with a shear rigidity that is lacking in simple bilayer vesicles (Takakuwa and Mohandas, 1988; Mohandas and Evans, 1994). In mammalian cells, this relatively flexible submembranous CSK allows the cell to maintain excess membrane surface area beyond that required to enclose its volume when the cell is not fully spread (i.e., it buckles when cells round like when fabric is pulled together with a purse string). This additional surface area serves as an immediate membrane reserve (Evans, 1992; Mohandas and Evans, 1994), such that when a mammalian cell experiences

rapid mechanical deformations (e.g., inflation, stretching) the excess membrane will first unfold and smooth out before significant tension develops in the lipid bilayer (Knutton *et al.*, 1976; Solsona *et al.*, 1998; Raucher and Sheetz, 1999; Zhang and Hamill, 2000). This view is supported by the finding that the level of membrane tension measured in animal cells using optical tweezers is 1000 times less than that which is required to activate MS channels in the membranes of bacterial cells (Vogel and Sheetz, 2006). Other studies suggest that the plasma membrane of animal cells cannot be stretched; instead new membrane needs to be exocytosed or membrane lipids need to flow from one place to the other during cell spreading or major cell shape changes to prevent membrane tearing (Sheetz *et al.*, 2006). For these reasons, it has been suggested that MS channels are not directly activated by changes in lipid bilayer tension in mammalian cells (Sheetz *et al.*, 2006; Vogel and Sheetz, 2006).

However, the mechanism of MS channel activation in higher cells remains controversial. For example, membrane tension is likely not uniform throughout the surface membrane, and it is possible that the low membrane tension measured in animal cells is an artifact of the optical tweezer measurement technique which cannot effectively measure forces >100 pN (Hamill, 2006). Migrating cells also can generate traction forces at their leading edge that not only induce elongation and stretching of the whole cell, but also cause ripping of the plasma membrane at the cell's rear trailing edge (Mayer *et al.*, 2004). Many cells also exhibit membrane tearing under physiological conditions *in vitro* and *in vivo* (e.g., in intestinal cells during normal peristalsis) (McNeil and Ito, 1989) and thus, membrane tension can clearly reach lytic levels in animal cells (Hamill, 2006). Importantly, the cellular tensegrity model provides a way to reconcile these ostensibly conflicting findings, as will be described below.

Although cytoskeletal and ECM components have been suggested to contribute to MS channel gating, their molecular identity remains unknown. Cytochalasin, which disrupts the connectivity of the internal actin CSK without depolymerization of F-actin in living cells (Schliwa, 1982), increases the mechanosensitivity of many MS channels in animal cells (Guharay and Sachs, 1984; Sokabe *et al.*, 1991; Small and Morris, 1994; Hamill and McBride, 1996). This observation suggests that actin microfilaments may normally suppress the level of tension transmitted to MS channels through the bilayer or the underlying submembranous CSK. This may occur either through microfilament-binding interactions that produce molecular conformational changes or by altering the level of preexisting isometric tension (prestress) maintained in this molecular network. Microtubules also contribute to touch sensitivity in nematodes and insects (Thurm, 1964; Hamill and McBride, 1996; Tavernarakis and Driscoll, 1997). However, disruption of microtubules using colchicine has little effect on MS channel activity in

skeletal muscle and in *Xenopus* oocytes measured using the patch-clamp technique (Guharay and Sachs, 1984), it also does not significantly alter tactile sensation in the cockroach (Kuster *et al.*, 1983). Thus, MS channel interactions with these CSK elements may vary considerably between different cell and tissue types.

Direct evidence relating to how certain MS channels are activated by molecules in the CSK and/or ECM is lacking (Hamill and Martinac, 2001). The basic idea of the tethered model is that proteins within these relatively rigid structural networks directly interact with the MS channel and that specific consensus regions or domains in the membrane channel mediate these interactions. For example, C-terminal cysteine-rich regions (Kanzaki *et al.*, 1999) and N-terminal repetitive ankyrin repeats (Walker *et al.*, 2000) in the intracellular domain of MS channels may mediate interactions with adaptor proteins of the CSK or with other membrane components. Alternatively, these domains might serve to localize or cluster MS channels at particular sites where mechanotransduction occurs, rather than to mechanically gate the channel directly. Another possibility is that the main function of cytoskeletal or ECM linkages is to alter the level of tension experienced by the MS channel within the lipid bilayer by absorbing mechanical stresses, and thereby modifying the time-dependence of channel adaptation (Ingber, 1997b; Hamill and Martinac, 2001).

IV. TENSEGRITY-BASED CELLULAR MECHANOTRANSDUCTION

Cells, tissues, and organs are constructed as interconnected structural hierarchies composed of self-stabilizing, tensionally prestressed networks known as tensegrity structures (Ingber, 1997b, 2006). The cellular tensegrity theory proposes that tensional forces are borne by cytoskeletal microfilaments and intermediate filaments and that these forces are balanced by interconnected structural elements that resist compression, most notably, internal microtubule struts and ECM adhesions; this creates a state of isometric tension or "prestress" that stabilizes the entire cytoskeletal lattice (Fig. 2B). However, individual filaments can have dual functions and hence bear either tension or compression in different structural contexts or at different size scales (e.g., rigid actin filament bundles bear compression in filopodia) (Ingber, 1997b, 2003b).

The tensional prestress that stabilizes the whole cell is primarily generated actively by the actomyosin apparatus within contractile microfilaments. Additional passive contributions to this prestress come from cell distension through adhesions to the ECM and other cells, osmotic forces acting on the

cell membrane, and forces exerted by filament polymerization. Intermediate filaments that interconnect at many points along microtubules, microfilaments, and the nuclear surface provide mechanical stiffness to the cell through their material properties, and their ability to act as suspensory cables that interconnect and tensionally stiffen the entire CSK and nuclear lattice.

This internal microfilament–microtubule–intermediate filament CSK is permeated by a viscous cytosol and enclosed by a differentially permeable surface membrane that is mechanically supported by a highly elastic submembranous CSK. This submembranous cytoskeletal network is itself organized as a tensegrity at the molecular size scale (Ingber, 2003b) with tensed spectrin molecules being balanced by compressed actin protofilaments and by interconnected regions of the noncompressible lipid bilayer (Fig. 2C) (Sung and Vera, 2003; Vera *et al*., 2005). Importantly, the efficiency of mechanical coupling between this submembranous structural network and the deeper internal CSK depends on the type of molecular adhesion complex that forms on the cell surface. Specifically, integrins that form focal adhesions and connect to the deep CSK can efficiently resist shape distortion, whereas other transmembrane receptors that only connect to the submembranous CSK distort easily when mechanically stressed (Fig. 3) (Wang *et al*., 1993). Thus, this cortical CSK can either act independently or in concert with the remainder of the cell and deeper microfilament–microtubule–intermediate filament lattice.

As a result of this integrated hierarchical architectural arrangement, forces applied at the macroscale that mechanically strain ECMs and deform cells and their internal CSK through integrins are able to filter down to smaller size scales and become focused on specific molecular components, both locally near the site of force application and at distant sites within the cell and nucleus. These focused stresses produce structural rearrangements within a subset of these molecules at the nanometer scale that changes their biochemical activities through kinetic or thermodynamic alterations (Ingber, 2006; Kumar *et al*., 2006). The mechanical stability of the network, and the efficiency of force transfer, can be changed by altering the level of prestress within the lattice; this can occur at one level of structural hierarchy (e.g., internal deep CSK independently of the submembranous CSK), or throughout structures distributed throughout the whole cell depending on the pattern of structural connections that the cell forms in response to different microenvironmental stimuli (e.g., whether it is bound to ECM via integrins or other cells via cadherins or selectins) (Wang *et al*., 2001; Ingber, 2003b).

As described above, the cellular tensegrity model is now supported by a plethora of experimental evidence (Caspar, 1980; Wang *et al*., 1993, 2001; Pickett-Heaps *et al*., 1997; Farrell *et al*., 2002; Hutchison, 2002; Hu *et al*.,

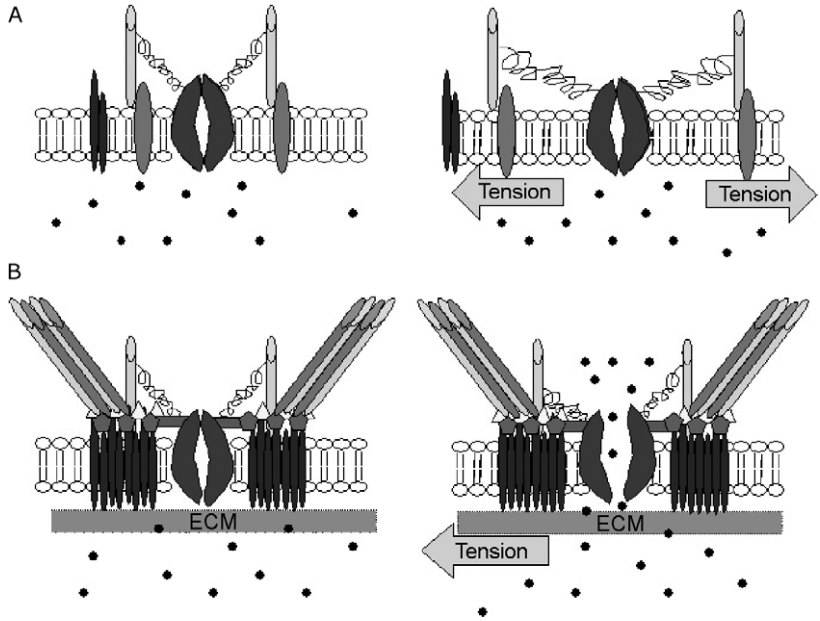

FIGURE 3 Schematic representations of force application to transmembrane surface proteins that connect only to the submembranous CSK (A) or to integrin receptors that form focal adhesions that physically link the ECM, membrane, and submembranous CSK to the deep CSK (B) in mammalian cells. (A) Forces dissipate locally due to the high flexibility of the submembranous CSK (e.g., through extension of spectrin molecules) and thus, MS channels do not experience levels of distortion (strain) required for their activation. (B) When forces are applied to integrins that form stiffened focal adhesions, these stresses are channeled through protein linkages to associated MS channels in the focal adhesion, thereby causing molecular distortion and MS channel activation. Disruption of deeper actin microfilament tethers would result in greater distortion of the entire focal adhesion complex, increased internal strain, and hence, greater distortion-based activation of the associated MS channels (not shown).

2003, 2004, 2005; Zanotti and Guerra, 2003; Brangwynne *et al.*, 2006; Kumar *et al.*, 2006) as well as by advances in mathematical, engineering, and statistical modeling (Connelly and Back, 1998; Stamenovic and Ingber, 2002; Wendling *et al.*, 2003; Lin *et al.*, 2004; Liu *et al.*, 2004; Sultan *et al.*, 2004; Shen and Wolynes, 2005; Sitharaman *et al.*, 2005; Vera *et al.*, 2005). In addition, a tensegrity-based computational model of the submembranous CSK of the red blood cell has recently been described which adds additional support for the concept of the cell being a hierarchical tensegrity structure in which this cortical CSK can function either independently or in concert with the remainder of the internal CSK depending on whether these two networks are mechanically coupled inside the cell.

This is important in the context of mechanotransduction because the hierarchical cellular tensegrity model predicts that key cytoskeletal anchoring molecules, such as integrins, may preferentially sense physical forces at the cell surface and transmit these mechanical signals through either linkages within focal adhesions or deeper filamentous CSK connections to other mechanochemical transducer components, such as MS channels, at various locations inside the cell. The use of tensegrity by cells would also suggest that the actin CSK may modify MS channel activity by controlling the level of isometric tension (prestress) within the regions of submembranous cytoskeletal network (i.e., when it is connected to the deep CSK), rather than through direct binding interactions between actin and the channel molecules. For example, an increase of prestress in the submembranous CSK resulting from transmission of tensional forces from the actin CSK via focal adhesion connections may locally suppress cortical membrane deformation and thereby "tune" MS channel activity much like increasing the level of tension in a violin string that constrains the vibrational displacement of the string and hence, alters sound propagation when the string is strummed (Ingber, 1997b).

Importantly, cellular tensegrity can reconcile the reality that animal cells have excess membrane which is much more flexible than bacterial membranes, yet forces can be distributed across wide regions of the cell surface (e.g., from the leading edge to the trailing membrane). Tensegrity predicts that these forces are not transmitted over long distances through the bilayer; rather they are channeled through integrins, focal adhesion proteins, and associated cytoskeletal filaments that connect one pole of the cell to the other, and link the surface membrane to the nucleus (Fig. 4). If the force is exerted on the membrane bilayer directly, then tearing will occur locally if the force produces a large hole in the membrane because it is exerted faster than membrane flow or replenishment can take place; this can occur at the initial site of force application, or at distant sites where forces are channeled through cytoskeletal connections (e.g., trailing edge of the cell).

In contrast, formation of integrin-mediated focal adhesions may overcome the high flexibility of surface membrane by recruiting focal adhesion proteins stiffening the cortical CSK locally (Fig. 3). Forces may then be focused on MS channels that associate with the stiffened focal adhesion, or with channels located at distant sites in the cell that are connected to the focal adhesion by stiffened linkages that channel these forces (Fig. 4), as has been shown to occur in many cell types (Maniotis *et al.*, 1997; Hu *et al.*, 2003). The clearest example of use of the discrete CSK channeling mechanism by cells is the demonstration that force application to cell surface ECM adhesions and associated cell distortion can activate MS channels on the nuclear membrane deep inside the cytoplasm (Itano *et al.*, 2003).

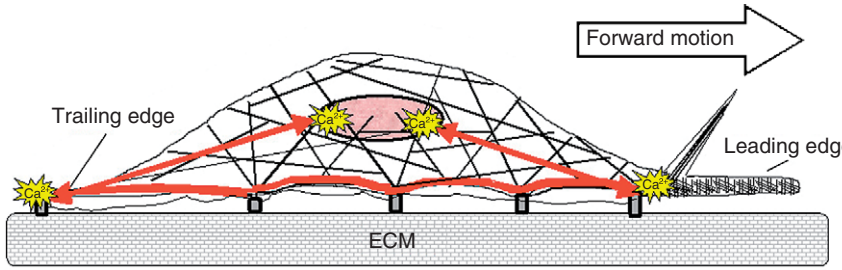

FIGURE 4 Schematic of a migrating cells showing both local and long-range activation of MS ion channels via force transfer through discrete cytoskeletal filaments. Migrating cells exert greatest traction forces on integrins within focal adhesions just behind the leading edge, and at the rear trailing edge of the cell, because tensional forces (red double-headed arrows) are transmitted through interconnecting cytoskeletal filaments (e.g., stress fibers that can span almost the entire length of the cell). Forces also can be transmitted over microfilaments and intermediate filaments to the nucleus at the cell center which commonly distorts in a coordinated manner as cells spread and move. Channeling of forces through the cytoplasm in this manner can result in stress concentrations simultaneously at multiple sites located throughout the cell that can activate MS channels and promote Ca^{2+} entry in these various locations (i.e., leading edge, trailing edge, and nuclear membrane). In this manner, animal cells can transmit tensional forces over large distances of the cell membrane through internal stiffened cytoskeletal elements, rather than through the lipid bilayer or the cortical membrane which could not support this type of force transfer to due its high flexibility.

This finding clearly shows that long distance force transfer which can activate MS channels at multiple locations in cells likely does not occur via lateral transmission through the bilayer, but rather through channeling via discrete filamentous connections inside the CSK (Fig. 4), as predicted by the tensegrity model.

V. FORCE TRANSMISSION THROUGH INTEGRINS IN LIVING CELLS

In living tissues, mechanical stresses are normally distributed to cells through the ECM scaffolds that hold the cells together and provide mechanical support to the tissue (Alenghat and Ingber, 2002). As described above, mechanical signals that propagate from the ECM converge on cell surface integrin receptors (Ingber, 1997b; Kumar *et al.*, 2006) that span the plasma membrane and physically link intracellularly to the contractile actin CSK by forming specialized macromolecular complexes, known as focal adhesions, that function as dynamic "spot welds" that anchor the cell to the ECM (Burridge and Chrzanowska-Wodnicka, 1996; Geiger *et al.*, 2001). The CSK

responds mechanically to forces transferred over the ECM and channeled through integrins by rearranging interlinked actin microfilaments, microtubules, and intermediate filaments that comprise the lattice, thereby strengthening the whole cell against the potential deleterious effects of mechanical distortion (Wang *et al*., 1993, 2001; Maniotis *et al*., 1997; Ralphs *et al*., 2002; Ingber, 2003b; Matthews *et al*., 2006). The ability of the elements of the cytoskeletal network to rearrange and stiffen in response to stress also depends on the level of tensile prestress in the CSK, in accordance with the tensegrity model (Ingber, 1997a, 2006; Stamenovic and Ingber, 2002; Stamenovic *et al*., 2003).

The use of transmembrane adhesion receptors and linked cytoskeletal filament networks for force transmission provides a way for cells to channel and focus stresses applied at the cell surface so that they concentrate on local focal adhesions as well as at distant sites in the cell (e.g., mitochondria, nucleus, focal adhesions at the opposite pole of the cell) (Wang *et al*., 1993; Davies *et al*., 1994, 2003; Maniotis *et al*., 1997; Helmke *et al*., 2003; Ingber, 2003b; Matthews *et al*., 2004, 2006). For example, when mechanical stresses are applied to specific cell surface receptors using ligand-coated magnetic microbeads with applied magnetic fields, the cell appears either highly flexible or extremely stiff when probed through transmembrane metabolic receptors (e.g., growth factor receptors, histocompatibility antigens) that only link to the submembranous CSK or through integrins that form focal adhesions that connect this cortical network to the deeper CSK (microfilament–intermediate filament–microtubule–nuclear lattice), respectively (Fig. 3) (Wang *et al*., 1993, 2001; Maniotis *et al*., 1997; Matthews *et al*., 2004).

Another pertinent example is that when cells that express constitutively active myosin light chain kinase (and hence produce higher prestress in the deep CSK) were compared with control cells using this magnetic cytometry method, no difference in cell stiffness could be detected when probed through transmembrane molecules that only link to the submembranous CSK, whereas the more highly prestressed cells appeared much stiffer than controls when probed through integrins (Cai *et al*., 1998). Importantly, when the stiffness of the same cells was analyzed using the classic glass pipette "poking" technique, the "cortex" appeared stiffer in the more highly prestressed cells (Cai *et al*., 1998). This means that poking produces extremely large mechanical strain in the surface membrane and nonspecifically (i.e., in the absence of specific focal adhesion linkages) distorts the deeper CSK as well. If this distortion is great enough, it could potentially activate MS channels or internal Ca^{2+} release mechanism by pulling on cytoskeletal elements that link to these structures from inside the cell.

Integrins can trigger signaling transduction cascades and induce focal adhesion formation as a result of ECM ligand binding and associated changes in integrin receptor conformation alone (Shimaoka and Springer, 2003; Springer and Wang, 2004). However, application of mechanical forces to bound integrins also can convey distinct biochemical signals to the cell (Ingber, 1997b; Geiger and Bershadsky, 2001). For example, application of force to integrins induces activation of Rho and its effectors mDia and ROCK, which promotes actin filament polymerization and induces cytoskeletal contraction, respectively; these effects result in focal adhesion formation (Riveline *et al.*, 2001; Galbraith *et al.*, 2002). Stresses applied to integrins can also regulate gene expression both transcriptionally by activating chemical signaling cascades (e.g., cAMP signaling; Meyer *et al.*, 2000) and posttranscriptionally by modulating the formation of protein synthetic complexes at focal adhesions (Chicurel *et al.*, 1998). In addition, mechanical stress application to cells already bound to ECM can induce a new wave of integrin activation which results in the activation of Rac and Rho that are important for lamellepodia extension, stress fiber reinforcement, and realignment of cells when these receptors bind to additional ECM molecules (Tzima *et al.*, 2001, 2002; Tzima, 2006).

Integrins also mediate many other effects of mechanical forces on biochemistry and cellular physiology. For example, cell proliferation depends on the ability of cells to spread and generate traction stresses on the ECM (Huang and Ingber, 1999), a process which is mediated through integrin-dependent activation of Rho, mDia1, and ROCK (Mammoto *et al.*, 2004). Strain-induced activation of p38 MAP Kinase in cardiomyocytes is integrin dependent (Kudoh *et al.*, 1998; Aikawa *et al.*, 2002), as is the release of growth factors by cyclic strain in vascular smooth muscle cells (Martinez-Lemus *et al.*, 2003). Furthermore, integrin-dependent cell spreading and associated mechanical distortion of the nucleus appear to induce Ca^{2+} entry into the nucleus and turn on gene transcription through activation of MS channels on the nuclear membrane in fibroblasts (Itano *et al.*, 2003). Thus, preferential channeling of forces through integrins, focal adhesions, and linked cytoskeletal networks that produce stress concentrations at numerous sites in the cell (Maniotis *et al.*, 1997; Wang *et al.*, 2001; Hu *et al.*, 2003, 2004, 2005; Ingber, 2006) may be responsible for simultaneously distorting, and thereby activating, multiple mechanotransducer molecules throughout the cell, as suggested by the tensegrity model (Ingber, 1997b, 2006). Importantly, experimental studies confirm that the efficiency of force channel through the CSK also depends on the level of prestress in the cell, such that only local stress concentrations are observed at the site of force application when prestress is dissipated using pharmacological or genetic techniques (Hu *et al.*, 2003).

VI. POTENTIAL LINKAGES BETWEEN INTEGRINS AND MS ION CHANNELS

Although it is now clear that integrins mediate various forms of mechanotransduction, their role in control of MS channel activity remains unclear. The possibility that integrins may also control MS channel function has been raised based on circumstantial evidence in the past. For example, mechanical strain-induced electrophysiological responses can be inhibited in chondrocytes by adding either soluble antibodies or RGD-peptides (from the integrin-binding site of fibronectin) that interfere with integrin binding, or the MS channel inhibitor, gadolinium chloride (Salter *et al*., 1997). Uniaxial cyclic strain also activates MS Ca^{2+} channels in endothelial cells (Naruse *et al*., 1998a,b; Sasamoto *et al*., 2005), and this is accompanied by increased expression of β3-integrins (Suzuki *et al*., 1997). Both integrin activation and Ca^{2+} influx are also critical for stretch-induced IL-6 secretion in endothelial cells (Sasamoto *et al*., 2005).

To explore whether forces applied to integrins induce changes in MS channel activity, investigators have applied mechanical forces directly to integrins via receptor-bound magnetic microbeads in conjunction with applied magnetic fields. Application of tensional force to surface-bound, collagen-coated microbeads with a magnetic tweezer induces a global increase in cytoplasmic Ca^{2+} levels in fibroblasts (Glogauer *et al*., 1995). However, it remains unclear whether MS channels directly mediate these effects, or if the applied stress induces release of Ca^{2+} from intracellular stores in these cells (Glogauer *et al*., 1995, 1997b, 1998). Interestingly, actin, but not vinculin, was recruited to the bead site in response to direct force application to these collagen-coated beads, and disrupting the actin CSK with cytochalasin D increased the integrin-mediated Ca^{2+} release in response to force application (Glogauer *et al*., 1995, 1997a).

Using a similar magnetic manipulation technique, we showed that application of force directly to integrins via bound magnetic RGD-coated microbeads that ligate cell surface integrins induces a rapid increase in intracellular Ca^{2+} levels in capillary endothelial cells and that this response can be suppressed by addition of the MS channel inhibitor, gadolinium chloride (Matthews *et al*., 2006) (Fig. 5). In more recent unpublished studies, we have detected local Ca^{2+} influx at the site of bead binding to integrins within 70 ms after force administration, as well as a force-dependent increase in Ca^{2+} entry as the level of stress was raised from 0.1 to 2 nN. Most importantly, application of similar forces to nonintegrin transmembrane receptors that do not promote focal adhesion formation on the surface membrane of the same cells did not produce this response, and similar responses were observed in multiple cell types. Thus, generalized deformation of the plasma membrane does

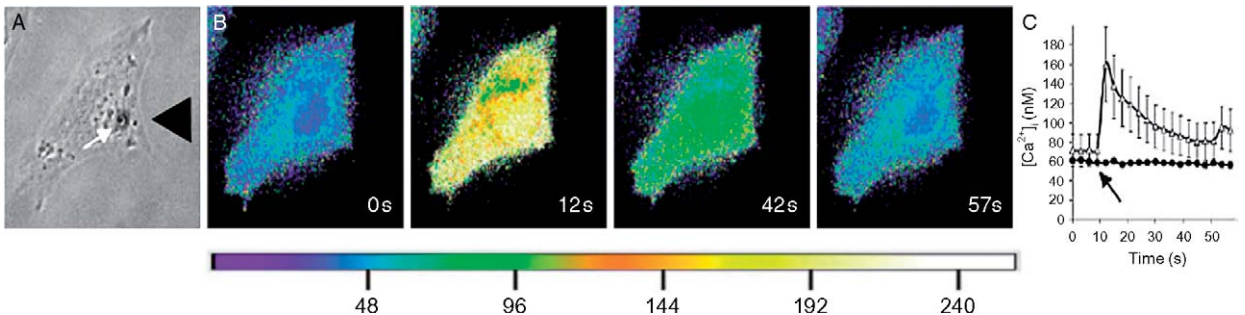

FIGURE 5 Activation of Ca^{2+} entry through MS ion channels by forces applied to cell surface integrin receptors using magnetic pulling cytometry. (A) Phase contrast view of an adherent capillary endothelial cell with attached RGD coated magnetic microbead (4.5 μm, white arrow). Black arrowhead denotes the position of an electromagnetic needle used to apply force to cell via the attached magnetic bead. (B) A time series of pseudocolored fluorescence images of the cell shown in (A) after mechanical stress (5 nN) was applied with the magnet. These pseudocolored images demonstrate a transient stress-induced increase in intracellular calcium $[Ca^{2+}]_i$ as a brief shift in color from blue to yellow over 0–57 s, as detected using FURA-2AM ratio-imaging (color bar indicates $[Ca^{2+}]_i$ in nanomolar; the force pulse was applied at 9 s). (C) Plot of average $[Ca^{2+}]_i$ for control (open diamonds) and gadolinium chloride-treated (solid circles) cells as a function of time; the inhibition of stress-induced Ca^{2+} influx by gadolinium suggests that force-induced Ca^{2+} entry into these cells is via stress-dependent activation of MS channels. Black arrow indicates when the 3-s force pulse was applied. Reprinted with permission from "The Company of Biologists" (Matthews *et al.* (2006)).

not appear to be sufficient to activate Ca^{2+} entry through MS channels in these cells; forces must be applied through activated (ligated) integrins that couple to the CSK through focal adhesions to activate this mechanochemical transduction response. The rapidity of the response also suggests that this mechanism likely occurs directly at the site of force application within the focal adhesion.

If this is true, then how do integrins gate MS ion channels? One possibility is that integrins may bind directly to certain MS channels or associate within common macromolecular complexes on the surface membrane, and there is some evidence to support this possibility. For example, ENaC channels and voltage-gated calcium channels (VGCC) coprecipitate in β1-integrin immune complexes isolated from mouse chondrocytes (Mobasheri *et al.*, 2002; Shakibaei and Mobasheri, 2003). Polycystin 1 (PC1), a component of the polycystin 2 (PC2) TRPP family channel complex that also associates with other members of the TRP family (Nilius and Voets, 2005), colocalizes with the collagen receptor, integrin $\alpha 2\beta 1$, in renal epithelial cells (Wilson *et al.*, 1999; Wilson, 2001). Integrin-associated focal proteins, such as vinculin and FAK, co-immunoprecipitate with PC1 as well (Wilson *et al.*, 1999; Wilson, 2001).

Interestingly, most of the TRP channels contain ankyrin domains (Minke and Cook, 2002; Nilius and Voets, 2005; Nilius *et al.*, 2005) which can bind to cytoskeletal adaptor proteins (Hryniewicz-Jankowska *et al.*, 2002). Intriguingly, ankyrin domains in integrin-linked kinase (ILK) appear to be important for its association with integrins in focal adhesions (Wu, 2004; Hannigan *et al.*, 2005). The focal adhesion protein kinase FAK also has been shown to directly associate with C-terminus of the hSlo α-subunit of the large conductance Ca^{2+}-activated potassium (BK) MS channel (Rezzonico *et al.*, 2003). Thus, taken together, these observations support the possibility that integrins interact with MS channel complexes in focal adhesions. Hence, external forces may activate MS channels as a result of being channeled across the cell surface through transmembrane integrin receptors, rather than as a result of generalized lipid bilayer distortion.

Given these observations, we need to readdress the gating of MS channels in the light of the previously proposed bilayer tension or tethered gate models (Fig. 1). TRAC1 channels that contain short ankyrin adapter domains can be directly activated by membrane stretch in reconstituted liposomes that are devoid of CSK (Maroto *et al.*, 2005). Other MS channels can be activated in membrane "blebs" that are torn free from underlying cytoskeletal connections also supporting the idea that these channels may be activated by force transfer through the bilayer; however, these channels do not exhibit the normal regulated behavior of channels observed in intact cells (Hamill and McBride, 1997; Hamill and Martinac, 2001). Thus, while forces

may be able to be transmitted through the bilayer, this may not be what is happening under physiological conditions.

In fact, TRAP1 activation appears to require multiple ankyrin domains that could mediate binding interactions with CSK proteins (Corey *et al.*, 2004) or integrins (Wu, 2004; Hannigan *et al.*, 2005). Also, while the newly cloned BK channels (Ca^{2+}-activated K^+ channels) from chick ventricular myocytes can be activated by changes of membrane tension induced by amphipaths, deletion of the STREX domain in the channel abolishes this response (Tang *et al.*, 2003; Qi *et al.*, 2005). Thus, the observed changes in channel gating are likely mediated by interactions with adapter proteins, rather than resulting from direct effects of amphipaths on the lipid bilayer alone (Tang *et al.*, 2003; Qi *et al.*, 2005).

Thus, there is strong evidence that ECM–integrin–focal adhesion (CSK) linkages might be crucial for gating of certain MS channels; however, the mechanism underlying this regulation is presently unclear. However, here we propose potential mechanisms that may mediate this response in eukaryotic cells as shown in Fig. 3.

As described above, eukaryotic cells generally contain excess membrane area relative to their volume such that many small membrane extensions are observed on the cell surface. This is possible because the submembranous CSK that provides most of the shape stability of the plasma membrane is highly flexible, and this is largely due to the great extensibility of the spectrin molecules that form the core lattice. If MS channels contain structural motifs that physically link them to this submembranous CSK, then they might experience deformation and change their gating activity when this lattice is mechanically stressed. However, because there is so much excess membrane and the submembranous CSK is so flexible, it is likely that the applied forces would dissipate through restructuring of the membrane before altering channel mechanics (Fig. 3A), except for high levels of mechanical strain or very rapid and highly focused perturbations, but these perturbations also may cause membrane tearing.

In contrast, when integrins are bound and activated, they form focal adhesions that must in some way physically integrate with portions of the submembranous CSK that is present throughout the cell cortex (Fig. 3B). The high density of tightly bound focal adhesion molecules will stiffen the associated portion of the submembranous CSK and thus focus stresses that propagate from the ECM, or from within the cell, on associated MS channel proteins in an integrin-dependent manner. These channels will experience a high local stress due to the increased rigidity of the lattice and enhance their gating activity. In contrast, application of the same force to other transmembrane proteins that do not associate with a rigidified focal adhesion (but still interact with the lipid bilayer) will not activate this response, again because

the stresses would dissipate within the flexible submembranous CSK lattice before they produced MS channel distortion. In summary, focal adhesion assembly might provide a way to rigidify structural linkages between integrins and MS channels, and thereby channel forces between these molecules. It also might recruit MS channels and thus increase the number of functional channels at these mechanosensing sites.

The finding that disruption of the actin CSK with cytochalasin D increases (rather than decreases) stress-dependent MS channel activation in many cells also needs to be readdressed in context of what we have learned about integrins and cellular tensegrity. First, it is important to clarify that cytochalasins do not cause F-actin depolymerization in intact cells; rather they produce breakage of the central actin network (Schliwa, 1982). In fact, when cut, the tensed actin filaments usually retract back to the cell cortex which appears to remain intact in cytochalasin-treated cells, and cells can adhere to ECM and form focal adhesions in the presence of high concentrations of cytochalasin (Ingber *et al.*, 1995, unpublished observations). However, disruption of the integrity of the actin CSK will dissipate prestress in the cell.

If MS channel activity is due to the level of isometric tension exerted on the protein, then lowering this prestress by disrupting the central actin CSK should decrease force sensitivity (i.e., increased stress would need to be applied to reach the same final state of tension that is necessary to produce channel activation). On the other hand, if MS channel activation depends on local mechanical strain in the rigidified focal adhesion/MS channel complex, then loss of prestress will increase channel activation in response to stress because greater local distortion of the focal adhesion will be produced when it is severed from the tensed internal CSK, much like when an untethered sail "luffs in the wind." Thus, MS channels may sense local mechanical strain that will be greater when forces are transmitted over the stiffened focal adhesion relative to the flexible submembranous CSK, and even greater when stabilizing tethers that connect the entire stiffened focal adhesion to the underlying contractile CSK are severed.

VII. CONCLUSIONS AND FUTURE IMPLICATIONS

Mechanotransduction is fundamental to many physiological responses and deregulation of this process leads to disease. MS ion channels constitute the first line of force transducers and regulate important functions such as hearing, touch, and vestibular function. However, both the identity of these channels and the precise gating mechanisms remain unknown in most cells. Force-dependent distortion of the lipid bilayer represents one potential way to gate MS channels, and this might occur in certain cells (e.g., in

some bacteria). But in specialized organs, such as the inner ear, MS channels are directly connected to force-bearing elements in the CSK, and their activity is sensitive to the level of prestress in the entire ECM–CSK lattice (Ingber, 2006).

Integrin receptors, by connecting the ECM to the CSK and resisting cell-generated forces, are perfectly poised to modulate mechanical force transfer through living cells. Thus, they are excellent candidates for controlling mechanical gating of MS channels. Integrins connect to the CSK through a number of focal adhesion adapter proteins, which relay chemical and mechanical signals into the cells through change in their conformation and binding kinetics. Importantly, there is increasing evidence to suggest that some MS channels associate with focal adhesions, and thus, may form part of this nanoscale mechanochemical signaling complex. Moreover, recent work from our laboratory has confirmed that direct force application to integrins and associated focal adhesions activates Ca^{2+} entry through MS channels, whereas application of the same force to other transmembrane channels that do not form focal adhesions fails to produce this response in the same cells. Thus, we believe that, at least in the multiple mammalian cells we have studied, generalized cortical membrane distortion is not sufficient to activate these MS channels and, instead, that this is an integrin-dependent mechanotransduction response. Focal adhesion formation may be viewed to enhance channel sensitivity by locally increasing the rigidity of this macromolecular complex and hence, more efficiently channeling stresses to these critical transduction molecules.

Future studies on the mechanism of MS channel activation in eukaryotic cells will, therefore, need to explore the role of links between MS channels, integrins, and focal adhesion proteins, as well as how the deeper CSK influences channel sensitivity and adaptation responses. This will likely require a combination of biophysical, electrophysiological, genetic, biochemical, and cell biological techniques. But to fully understand this mechanism, it probably will be necessary to develop entirely new methods that will permit us to analyze single channel activities in the normal structural context of whole living cells, rather than within isolated membranes or regions of the intact membrane that are locally fixed to a rigid glass pipette (i.e., as is done with patch-clamp approaches now). Only this way, will it be possible to tease out the critical local and global structural elements that govern stress-dependent activation of MS ion channels in living cells.

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