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The "Stressful" Life of Cell Adhesion Molecules: On the Mechanosensitivity of Integrin Adhesome

Cells have evolved into complex sensory machines that communicate with their microenvironment via mechanochemical signaling. Extracellular mechanical cues trigger complex biochemical pathways in the cell, which regulate various cellular processes. Integrin-mediated focal adhesions (FAs) are large multiprotein complexes, also known as the integrin adhesome, that link the extracellular matrix (ECM) to the actin cytoskeleton, and are part of powerful intracellular machinery orchestrating mechanotransduction pathways. As forces are transmitted across FAs, individual proteins undergo structural and functional changes that involve a conversion of chemical to mechanical energy. The local composition of early adhesions likely defines the regional stress levels and determines the type of newly recruited proteins, which in turn modify the local stress distribution. Various approaches have been used for detecting and exploring molecular mechanisms through which FAs are spatiotemporally regulated, however, many aspects are yet to be understood. Current knowledge on the molecular mechanisms of mechanosensitivity in adhesion proteins is discussed herein along with important questions yet to be addressed, are discussed. [DOI: 10.1115/1.4038812]

Keywords: focal adhesions, mechanosensitivity, force transmission, signaling, proteins

The Mystery of Mechanosensitivity: How Mechanical Stimuli Affect Biological Processes?

Recently, significant evidence has emerged demonstrating that external mechanical forces, such as fluid shear stress in the vasculature or contractile force of cells' own actomyosin cytoskeleton, are critical determinants of the form and function of cells, tissues, and organisms [1,2]. This process is generally referred to as mechanotransduction and is due to the mechanosensitive functional and/or structural changes at the cellular, subcellular, and molecular levels [1,3,4]. Traditionally, biological regulation has been understood from the principles mediating solution biochemistry, including diffusivities, binding affinities, and reaction rates. Thus, most early work on mechanosensitivity focused on determining how mechanical stimuli could affect these processes [5,6]. An organizing principle has emerged that combines fundamental principles in biochemistry, that protein structure dictates protein function, and biophysics, that protein structure is largely dictated by multitude of rather weak interactions that are readily rearranged. Thus, the primary origin of mechanosensitivity is that applied forces induce conformational changes in protein structure as shown by generic protein 1 in Fig. 1 [2]. Similarly, force-induced conformation switching of focal adhesion (FA) proteins is required for regulating new binding events as illustrated by the interaction between mechanically activated proteins 1 and 2 in Fig. 1. For instance, the mechanically regulated association of proteins has been observed for several key structural proteins, including vinculin and talin, vinculin and α-catenin, as well as filamin and FilGAP [7–9]. Also, force can significantly affect signaling cascades, as force-induced conformation changes is required for a phosphorylation of p130CAS by Src-family kinases as well as the localization of MAP kinase 1 [10,11]. If the applied force on mechanosensitive proteins does not cause any alterations in the molecular composition, it may cause reinforcement or weakening of existing interactions. Important examples are the catch-bond-behavior of pairwise interactions between integrin, and ligands [12], as well as the interaction of actin with myosin

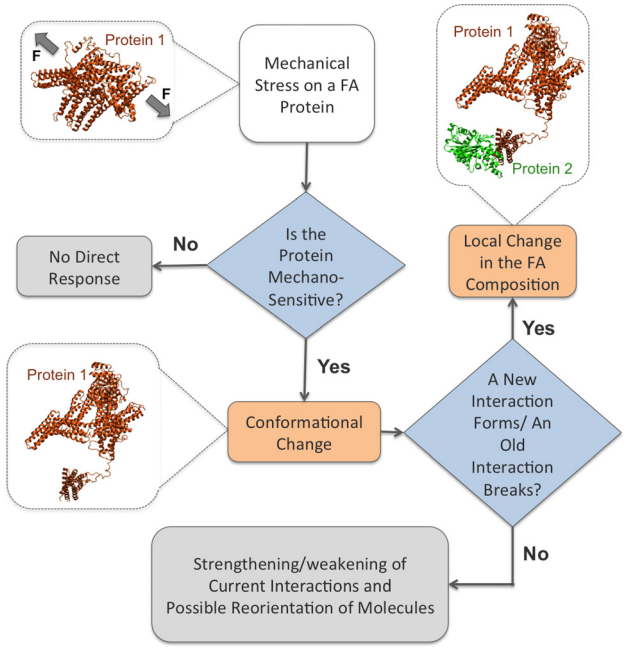

Fig. 1 Mechanosensitivity of FA proteins regulates the FA architecture. As mechanical stress impinges on a protein, the molecule responds by undergoing a conformational change. This may result in formation of new interactions or disruption of existing interactions, which modifies the local composition of the FA complex. Otherwise, the new conformation of protein regulates the strength of its existing interactions, e.g., catch bond formation.

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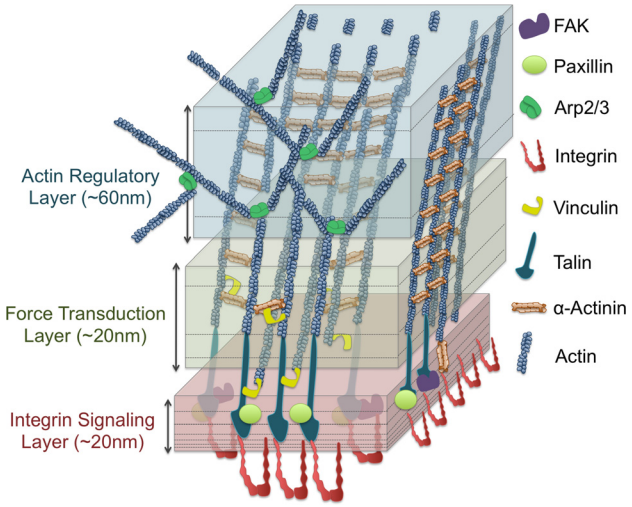

Fig. 2 The architecture of FAs. FAs can be divided into three functional layers each having a distinct molecular composition. Integrin receptors reside in the lipid membrane and are activated via binding to the talin head within the integrin signaling layer. Other important signaling molecules such as FAK and paxillin also function in the integrin signaling layer. The force transduction layer is rich in vinculin and the rod domain of talin, which is oriented toward actin. Actin and α-actinin are localized within the actin regulatory layer. The distal end of FA connects with the lamellipodial dendritic actin.

[13], α-catenin [14], and vinculin [15]. Furthermore, the cell is a mechanically integrated structure, as various subcellular organisms are linked through the cytoskeleton [16]. Thus, even the local application of forces can affect mechanosensitive processes throughout the entire cell and in certain cases [17], across multiple cells [18]. Thus, mechanochemical signal transduction can occur across different scales, and is particularly potent regulator of many fundamentally important cellular processes [4,19].

The Focal Adhesion: A Prototypical Mechanosensitive Structure

Cells associate with their microenvironment through large multiprotein complexes known as integrin-mediated FAs or the integrin adhesome [20], which are essential for many important cellular functions such as migration, proliferation, differentiation, and growth [21]. FAs are mechanosensitive subcellular organisms that consist of more than 150 proteins, which collectively form a mechanical linkage between the extracellular matrix (ECM) and the actin cytoskeleton [22–24]. FAs are part of a powerful intracellular machinery orchestrating mechanotransduction pathways, i.e., underlying transducing environmental signals to biochemical cascades [25,26]. Recent work using super-resolution microscopy with enhanced resolution in the vertical direction has shown that the proteins within the mature, long-lived FA are arranged in weakly stratified structures [24]. Specifically, these structures can be divided into three layers each having a distinct molecular composition and signaling features as shown in Fig. 2 [24,27]. The integrin signaling layer is the closest to the plasma membrane and consists of integrin regulatory proteins including FAK and talin's head domain [28]. Most of the proteins in this layer engage with the integrin cytoplasmic tail within a thickness of about 20 nm [27]. Mechanical signals are further transmitted to the force transduction layer, which is an intermediate segment between integrin signaling and cytoskeletal layers and contains mechanosensitive proteins such as vinculin and talin's rod domain [27]. The third layer, located 40 nm away from the plasma membrane, is the actin regulatory layer where both actin and its binding proteins such as α-actinin and vasodilator-stimulated phosphoprotein reside [27]. However, mechanisms and key players involved in regulating formation and maintenance of striated layers of FAs have not yet been fully understood.

In general, the mechanosensitivity of FA entails dynamic changes in this structure. For instance, despite the general consensus described about, the exact position of proteins depends on the mechanical stresses experienced by the FA [24]. For instance, the mechanical linker protein, vinculin, exists closer to the integrin signaling layer in newly formed contacts between cells and the ECM, and relocates to the force-transduction layer in response to cell contractility [28]. Similarly, other proteins, such as the actin regulatory protein zyxin selectively localize the mature FAs subject to significant stresses [29], and the localization of α-actinin has been shown to be dependent upon the type of ECM cells they are exposed to [30]. As discussed in further detail later, this dynamic mechanosensitivity has significant consequences for both the transmission of force across FAs as well the signaling pathways initiated at the FA.

Where Do Forces Come From?

Stresses within FAs may originate from both intra- and extracellular sources [31,32]. Differing cell type experiences a wide range of mechanical microenvironment characterized by a variety of mechanical cues [33]. For instance, the endothelial cells, which line the walls of blood vessels, are constantly exposed to pulsatile blood flow. Additionally, smooth muscle cells situated underneath the endothelial cells monolayer only experience the shear stress from the interstitial flow, which is much lower than that of the blood flow applied on ECs [34,35]. External loads within the ECM are transmitted to cells through integrin receptors specific to the ECM ligands [36]. Cellular traction forces are produced by actomyosin contractility, which pulls on the ECM [37–39].

Cell migration requires high traction forces against the substrate, which is generated via actomyosin contraction [38,40]. FAs transmit cytoskeletal forces and prevent slippage between the cell and the substrate by directly associating with the extracellular ligands [41]. As suggested under the molecular clutch hypothesis, the FA acts as a clutch between the rearward actin flow and the ECM as shown in Fig. 3 [24,41,42]. It has been shown that cells adjust the strength of their adhesions to the stiffness of the substrate [36,43]. This also showed that the clutch hypothesis does not suggest a rigid behavior as the two ends of the engaged molecular clutch undergo different flow rates [24,44,45]. This is also consistent with the distinct molecular connections formed at different stages of FA formation and maturation [30,46]. Such spatiotemporally varying coupling dictates the various responses of FAs to applied load [47]. If nascent adhesions does not fully engage with the actin retrograde flow, they undergo quick turnovers (~1 min) [48]. Myosin II contractility is required for further maturation of FAs, resulting in growth along the direction of the actin flow [39,48,49]. Mature FAs remain attached to stress fibers throughout their lifetime, and their maintenance requires association with contractile F-actin bundles [50,51].

Mechanosensitivity Across Multiple Scales in Focal Adhesions

Signaling across different scales is mediated by mechanosensitive interactions, which links molecular events to cellular phenotypes [16]. FAs also exhibit distinct behaviors on different length scales. For instance, FA proteins exhibit flow-like patterns that are driven by the dynamics of the cytoskeleton [45]. Specifically, the coupling between the flow of different FA molecules and myosin-mediated retrograde flow most likely occurs through mechanosensitive proteins. The local force distribution within each FA layer is determined by the speed of the actin retrograde flow as well as the molecular composition in that layer, which then selectively attract certain molecules [27,40]. As proteins are

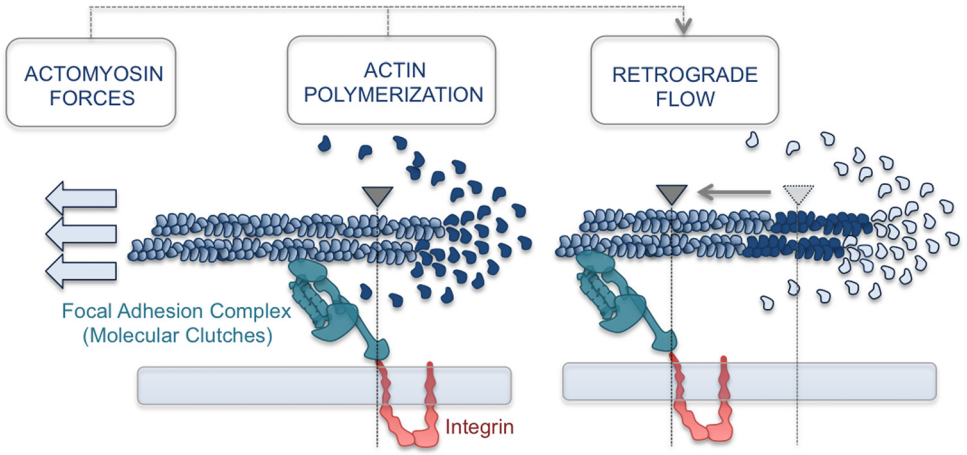

Fig. 3 The retrograde flow of actin. Actin polymerizes at the cell edge, while actomyosin forces are applied to the rear end of actin fibers. The combination of these effects results in a rearward flow of actin relative to the cell edge. The actin retrograde flow is transmitted to the ECM in the form of traction forces via FAs, which act as a "molecular clutch."

integrated into each layer, their speed becomes similar to the bulk of that layer [27,41]. For instance, the flow of α-actinin has been observed to be very similar and strongly coupled both in terms of speed and direction with the actin retrograde flow demonstrating direct association with actin [27]. The flow of integrin has the lowest coupling with the actin flow due to the direct binding between integrin and the ECM molecules, while flows of vinculin and talin rod show relatively higher couplings with the actin flow as they are localized within the force transduction layer and thus are closer to the actin regulatory layer. Thus, variable molecular-scale mechanical coupling to the cytoskeleton induces large-scale mechanosensitive dynamics within FAs.

Potentially consistent with the presence of multiple molecular linkages that behave as catch bonds, although multiple other processes may be involved, FAs also become reinforced under tension, a phenomenon referred to as adhesion strengthening [52]. The composition of early adhesions are significantly changed upon increasing local forces and eventually each FA plaque applies traction forces ranging from 1 to 10 kPa over an area of 1 μm^2 [53], whereas the load on individual proteins is in the order of 1–10 pN [54,55] as shown in Fig. 4. After FAs become mature and stable, individual molecules still undergo dynamic turnovers. This is a well-known characteristic of a system in a thermodynamic equilibrium in which macroscopic properties stay constant, while the microstate of the system dynamically changes [56]. Specifically, the life-time of FAs is in the order of several minutes, whereas according to the fluorescence recovery after photobleaching (FRAP), the time scale for the kinetics of diffusion (or flow) of most FA proteins such as FAK, talin, zyxin, and α-actinin is in the order of tens of seconds (shown in Fig. 4) suggesting that FA components are rapidly exchanged with cytosolic proteins [22,54,57–59].

Molecular Mechanosensitivity

Mechanosensing is mediated by force-induced changes either in protein conformations and/or in the kinetics of assembly and disassembly of protein complexes [60,61]. A common form of regulation amongst many mechanically relevant proteins is based on conformation [27,62]. In an "inactive form" the protein assumes a conformation that blocks binding to other proteins or sub-cellular structures to maintain a cytosolic distribution, as in vinculin and talin [63,64]. Often, this is mediated by the two ends of the protein and referred to as the head-tail inhibition. As adhesion strengthening involves the recruitment of proteins from the cytosol, a conformation change is often required to mediate incorporation into subcellular structures. A conformational change in a protein is a biased thermodynamic process in which thermal energy of the solvent molecules is driven by mechanical stress to produce a new structural state [65]. Mechanical stimuli may also change the free energy profile of the system and thus affect the probability of reaching a previously inaccessible state [2]. Identifying and detecting different conformational states of proteins as well as measuring molecular forces are modern challenges of mechanobiology, and various types of tools are currently being designed for these purposes. For instance, Tension and

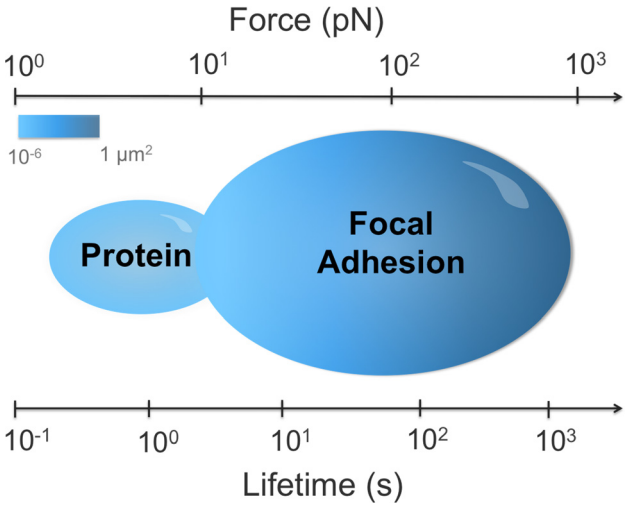

Fig. 4 The force and lifetime of FA and its components. The lifetime of proteins within the FA structure is in the order of seconds, while FA as a subcellular organism remains stable for several tens of minutes. Early adhesions only consist of a few proteins and last for tens of seconds. As they grow into focal complexes, their lifetime increases to a few minutes. The force that can trigger a mechanical response in a single protein is in the order of 1 to 10 pN, whereas forced exerted by FAs on the substrate is 2–3 orders of magnitude higher. The shade in shapes represents the area of the system, e.g., the FA area increases by force. The area of a single protein is roughly estimated to be in the order of 1 nm^2. The bar on the left side of the plot shows the shade scale used for the area in μm^2. The left shape representing the "protein" is illustrated as a droplet merging into the "focal adhesion" shown as a larger drop on the right.

conformation sensors based on flourescence resonance energy transfer (FRET) are important tools for detecting mechanical variables, namely conformational switching and load, and the correlation between these variables and regional dynamics in cells [66,67].

Although all mechanosensitive molecules and the exact stress distribution across various regions of FA have not yet been identified, mechanisms of mechanosensitivity of known molecules provide important insights into the local stress environment. It is important to note that mechanical deformation of a molecule depends on both its mechanical properties and the stress field. For instance, due to the direct anchorage of integrin receptors to the ECM molecules, proteins within the integrin signaling layer are under high frictional tension [16,68,69], i.e., high stress and low mobility. Pure electrostatic interactions may also give rise to conformational switching. For example, before engaging with integrin, talin exists in an auto-inhibited conformation such that the tail domain masks the β-integrin binding site on its head domain [70]. It is not quite clear how talin becomes activated prior to integrin binding, however, one proposed mechanism involves phosphotidylinositol-4,5-bisphosphate (PIP2) association, in which PIP2 induces the open conformation via a "pull-push" mechanism [71]. Specifically, the positive charges on the four-point-one, ezrin, radixin, moesin domain on the talin head is attracted toward PIP2, while negative charges on the rod domain are repelled from the membrane like a tug-a-war [71], which creates a local stress field proximal to the membrane.

Visualizing the Forces Experienced by Proteins and Conformational Changes: FRET-Based Tension Sensors. With the recent development of FRET-based tension sensors, it is now possible to measure the spatial and temporal variation of the forces experienced by specific proteins inside living cells [72,73]. These biosensors take advantage of the strong distance dependence of FRET to measure displacements within, and thus tension across, specific proteins of interest. Most tension sensors are based on a module, composed of two fluorophores separated by an elastic linker that can be inserted into a target protein. As a load is applied across the protein, the donor and acceptor fluorophores separate, and FRET decreases. By measuring the spatiotemporal distribution of loads through individual proteins in FAs and other cytoskeletal structures, one can start to unravel the mechanisms underlying mechanically sensitive phenomena in FAs. A vinculin tension sensor was used to establish that vinculin load bearing is a switch that dictates whether FAs assemble or disassemble in response to applied loads [54]. More recently, integrin tension sensors have been created and used to study force-induced integrin activation [74]. Furthermore, tension sensors implemented in three actin binding proteins, α-actinin, spectrin and filamin, reflected a direct relation between the stress level across these molecules and cell adhesion with the substrate [75]. Of note, is a pair of talin tension sensors were used to show that the tension within talin dissipates within in the protein [76]. The rod domain of talin has several vinculin binding sites (VBSs) that are inhibited under a low-tension condition [64,77,78]. As the actomyosin forces are applied on the talin molecule, the rod domain is extended and VBSs are exposed [55]. This raises the possibility that the stress within FAs may not be constant, and that different protein, or even regions of proteins, could be differentially loaded and exhibit distinct mechanosensitive properties. Furthermore, conformation sensors have demonstrated that not all proteins within a FA are activated, and this might have differential connections to the force-generating actomyosin cytoskeleton [24]. These results could partially explain the large variability in the molecular loads experienced by mechanical linker proteins in FAs [55,79] even if actomyosin forces are assumed to be locally constant. The comparison of tension sensors and conformation sensors in dynamic FAs, as is possible for vinculin, will likely lead to an enhanced molecular-scale understanding of mechanotransduction.

Mechnanosensitivity in Adherens Junctions

Forces generated in the actin cytoskeleton are transmitted across transmembrane receptors to other cells through cell-cell adhesions [80]. These cell–cell contacts act analogous to FAs in terms of transmitting forces to the extracellular environment in diverse cellular processes [24,81]. Specifically, similar molecular mechanisms of mechanosensitivity have been observed in cell–cell junctions. For instance, β-catenin directly binds to cadherins in cell–cell adhesions, which is similar to the binding between the head domain of talin and integrin in FAs [82,83]. Furthermore, vinculin binding to α-catenin is loosely analogous to the force-dependent recruitment of vinculin to the rod domain of talin [7,9]. Thereby, talin seems analogous to α- and β-catenin. As cytoskeletal forces necessary for unwrapping the VBSs are applied to α-catenin and talin, the likelihood of vinculin binding increases as shown in Fig. 5. Furthermore, it was shown that forces larger than 25–30 pN result in denaturing of these proteins and potentially prevented vinculin binding [9]. Additionally, tension and conformation sensors have been developed for both talin and α-catenin, potentially enabling future studies correlating load and conformation change in these molecules [55,84].

Existing Challenges and the Need for an Integrated Approach. Defining mechanosensitivity in adhesome components is difficult as various influential factors are involved including intrinsic structural states and environmental factors such as local ion concentration and stress distribution, which are difficult to measure. For instance, it is not yet clear how some protein–protein interactions act as catch bonds (i.e., reinforce under tension), while others show a slip-bond behavior (i.e., break under tension) [85,86]. Furthermore, the range of applied tension at FAs along with composition dependency of FA mechanosensitivity suggests diverse mechanisms by which individual molecules respond to force. Moreover, mechanical response of an individual protein most likely depends on the amount, duration, and direction of applied force and may vary upon the environment, e.g. one or more "mechanically activated" structures may exist to facilitate environmental adaptation. Furthermore, it is still unclear how forces are propagated within a single protein structure, e.g. how binding on one side allosterically alters distal parts of the structure

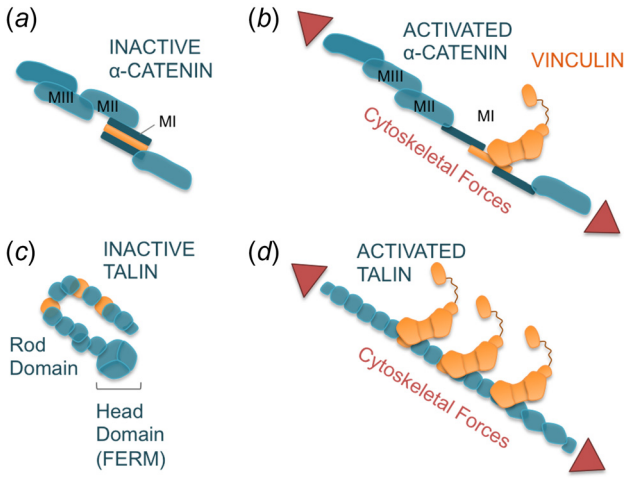

Fig. 5 Modularity of molecular mechanosensitivity. There are similar mechanisms of vinculin recruitment to cell-cell and FA contacts. (*a*) The VBS of α-catenin is inhibited inside the MI domain. (*b*) The cytoskeletal forces along α-catenin stretches the molecule and unravels the inhibited VBS. (*c*) Talin has 11 VBSs along its rod domain, which are inhibited in the absence of mechanical stress. It should be noted that only three VBSs are shown for simplicity. (*d*) Tension along talin's rod domain increases its affinity for vinculin binding.

[87]. Having the detailed spatial organization of FA molecules is important to understand direction and amount of forces acting on each molecule [28]. Therefore, highly structure-based approaches are needed to understand and characterize mechanosensitivity at both molecular and subcellular levels.

Rapidly growing structural data combined with state-of-the-art molecular simulations serves as a powerful tool for studying load and conformation of mechanosensitive proteins. Such knowledge can be used to inform studies involving FRET-based biosensors, which often envision load and conformation as binary states (open versus closes or loaded versus unloaded). The mechanosensitive intermediate states can be explored using molecular simulations; however, modeling per se is not able to show the existence of these states in cells and their role in determining cell function. Thereby, it is critical to use FRET-based tension and conformation sensors to identify intermediate states in vivo and match them with computational observations. FRET-based tension sensors are essential for estimating the level of intra- and inter-molecular forces [55,88], while conformation sensors may report key information of the functional state of a protein. However, molecular dynamics simulations suggest that vinculin and likely other mechanosensitive proteins exist in a variety of mechanical intermediaries. Developing methods that translate the signals expected from these intermediate states in the existing sensors or developing new sensors that are directly capable of probing these intermediary states will be an important problem in the future. Furthermore, force transmission within the structure of proteins depends on both the constituent domains and their arrangement. It is important to examine domain-based mechanosensitivity in FA proteins in order to understand how forces mediate structural states of full-length proteins. This will aid in developing modular approaches for exploring mechanical properties of proteins, which can further be employed in characterizing newly discovered mechanosensitive proteins. Moreover, it may give new insights into engineering novel proteins to be used for measuring intramolecular forces and rescuing phenotypes resulted from defective mechanical responsiveness. Furthermore, a comprehensive understanding of domain-dependent mechanosensitivity may account for potential redundancies in function.

Conclusions

Cellular mechanotransduction is a multiscale/multiphysics process that is mediated by mechanosensitivity of participating proteins within complex cellular pathways that connects the extracellular matrix to the cytoskeleton and ultimately to the nucleoskeleton. One of the important gaps in understanding regulatory mechanisms of integrin-mediated mechanotransduction pathways is force-regulated conformational changes and intermediate states of individual proteins as well as the full protein–protein interaction patterns. Furthermore, interactions between different domains of FA proteins can serve as means of modularity, which controls how stress is captured within the protein structure. For instance, α-actinin, an actin cross-linker, undergoes stretching, twisting, bending, or a combination of these mechanical deformations depending on the local stress environment, which is modulated by its interdomain interactions [89–91].

In summary, FAs are dynamic mechanosensitive molecular complexes consisting of various proteins, which orchestrate force transmission between the ECM and the actin cytoskeleton. Structural and functional features of FA molecules are unique and have adapted throughout the evolution to sense and respond to mechanical stimuli. When mechanosensitive proteins are stressed, their conformation and binding affinities alter. However, the mechanism by which mechanical properties of adhesome components translate into FA formation and maturation has remained largely elusive. Thereby, a mechanistic, systems biology approach for tracking forces within the structure of proteins is essential for understanding the molecular basis of mechanosensitivity in FAs. Such knowledge will also provide an important insight into the specific role of individual proteins in FA regulation and potential redundancies in protein function, which will be highly beneficial to the field of mechanobiology.

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