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On the Molecular Basis for Mechanotransduction

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Abstract: Much is currently known about the signaling pathways that are excited when cells are subjected to a mechanical stimulus, yet we understand little of the process by which the mechanical perturbation is transformed into a biochemical signal. Numerous theories have been proposed, and each has merit. While cells may possess many different ways of responding to stress, the existence of a single unifying principle has much appeal. Here we propose the hypothesis that cells sense mechanical force through changes in protein conformation, leading to altered binding affinities of proteins, ultimately initiating an intracellular signaling cascade or producing changes in the proteins localized to regions of high stress. More generally, this represents an alternative to transmembrane signaling through receptor-ligand interactions providing the cell with a means of reacting to changes in its mechanical, as opposed to biochemical, environment. One example is presented showing how the binding affinity between the focal adhesion targeting domain of focal adhesion kinase and the LD motif of paxillin is influenced by externally applied force.

1 Introduction

Studies during the past two decades have illustrated the wide range of cellular responses to mechanical stimulation. Much of the impetus for this work stems from the discovery that stresses experienced in vivo are instrumental in a wide spectrum of pathologies. One of the first diseases found to be linked to cellular stress was atherosclerosis, where it was demonstrated that hemodynamic shear influences endothelial function [Dewey, Bussolari, Gimbrone, and Davies (1981)], and that conditions of low or oscillatory shear stress are conducive to the formation and growth of atherosclerotic lesions [see reviews (Davies (1995), Lehoux and Tedgui (2003))]. Even before then, the role of mechanical stress on bone growth and healing was widely recognized, and since then, many other stress-influenced cell functions have been identified. Collectively, this work has evolved into a field of its own, sometimes referred to as "mechanobiology".

Many have investigated the signaling cascades that become activated as a consequence of mechanical stress, and these are generally well characterized. The initiating process, however, by which cells convert the applied force into a biochemical signal, termed "mechanotransduction", is much more poorly understood, and only recently have researchers begun to unravel some of these fundamental mechanisms. Several theories exist that might explain the process of mechanotransduction, but most are still in their infancy. For example, it is known that the cell membrane contains stretch-activated ion channels that change conductance as membrane tension is increased. While many such channels have been identified, the atomic structure of only a few is known, and molecular dynamics simulations have proven somewhat inconclusive. It is also widely appreciated that proteins, in addition to transmembrane channels, undergo conformational change when subjected to stress. Such conformational change can alter binding affinities to other proteins, but might also initiate enzymatic activity, which has the attractive potential of leading to signal amplification. It is straightforward to demonstrate that the forces experienced by single proteins at the threshold of mechanotransduction are above the level of thermal noise and of sufficient magnitude to induce such changes in conformation or even bond rupture. Finally, we also know that changes in the intracellular distribution of structural proteins occur rapidly under the action of external force. All this points to the role of force-induced conformational change as a primary mechanism

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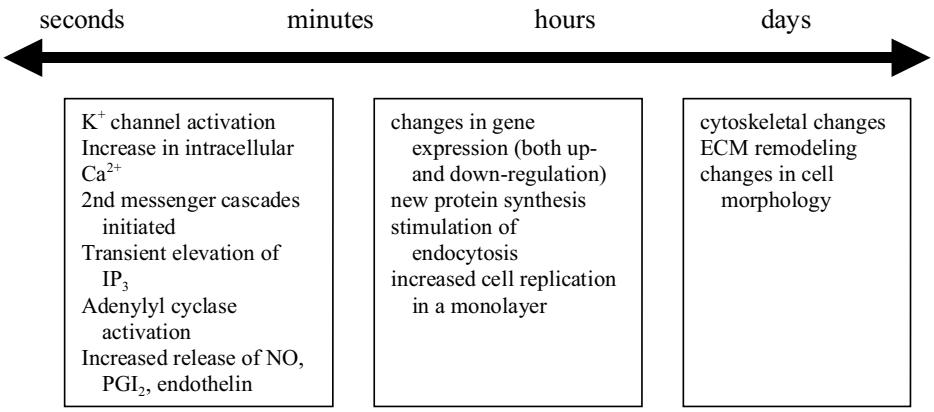

Figure 1 : Time scales for a subset of the numerous biological consequences of mechanical stimulation. Most short-term events are locally-mediated whereas the long-term consequences occur primarily as a result of altered gene expression.

of mechanotransduction, yet the evidence remains inconclusive.

One purpose of this paper is to summarize our current understanding of the role of protein conformational changes in the sensation of force by a cell, and at the same time, identify critical gaps in our knowledge or barriers to further understanding. We focus on endothelial cells as they have been extensively characterized in the literature, but also draw upon some results from other cell types in certain examples.

2 Nature of the Cellular Response

We have come to appreciate that cells are exquisitely sensitive to force, and respond in ways that give rise to changes in intracellular structure, migration, gene expression ad protein synthesis. There is also growing evidence that stress affects many post-translational changes in protein synthesis, and ultimately influences the composition of the surrounding extracellular matrix.

Cellular responses have been variously characterized, but it is particularly useful to consider the responses to mechanical stimulus in terms of their time scales and whether or not the response is mediated through changes in gene expression. In many respects, these divide responses along similar lines since the early responses occur long before it is possible to engage nuclear events, and the changes that occur on a longer time scale are generally those that pass through a more complex signaling cascade, and these often involve phenotypic alterations (see Figure 1).

Responses can be essentially instantaneous, with some early events, mostly transient changes in intracellular ion concentration, occurring within a millisecond time frame following the application of force [Lehoux and Tedgui (2003)]. It is likely that these earliest events are associated with changes in ion channel conductance, and consequently, fall into the category of stretch-activated ion channel activation. We know, however, based on experiments on cells for which the membrane has been removed by surfactant treatment, that cell stretching can alter the binding of several proteins to a focal adhesion complex [Sawada and Sheetz (2002)]. Although the time-scale for these events is not known, if they arise from changes in the molecular conformation of focal adhesion proteins, they could occur essentially simultaneous with the application of force. The most likely explanation for these experimental findings is a local conformational change instigated by cellular stretch, that produces a change in binding affinity for other cytoskeletal or focal adhesion proteins. Other responses, as seen in Figure 1, stretch over a period of hours and even days. Most of these, changes in cell morphology, for example, involve multiple signaling pathways and are mediated by both local transduction events and longer-term changes in gene expression.

3 Force transmission pathways within the cell

Davies [Davies (1995)] first put forth the concept that the initiating transduction event need not occur at the site of

force application to the cell. He described how forces, such as shear stress at the apical membrane of endothelial cells, could be transmitted via the intracellular structures, to remote points within the cell – cell-cell junctions, focal adhesions at the basal surface, nuclear membrane, the cytoskeletal proteins themselves – and initiate signaling there. While, in general, one would expect the levels of force to diminish as one moves further and further away from the site of force application, the nature of the cytoskeletal structures and their arrangement within the cell, such as their concentration at focal adhesion and other junctional complexes, could concentrate the force. For example, a distributed fluid shear stress acting at the apical membrane must be balanced by forces that tend of be more highly localized on the basal surface. Hence, stress levels in and around focal adhesions tend to be considerably larger than the hemodynamic stresses, by orders of magnitude if one considers that focal adhesions occupy no more than 1-10% of the basal cell membrane. See Mack et al. [Mack, Kaazempur-Mofrad, Karcher, Lee, and Kamm (2004)] who have described such stress focusing in the case of force application to endothelial cells by magnetic beads, and Hu et al. [Hu, Chen, Fabry, Numaguchi, Gouldstone, Ingber, Fredberg, Butler, and Wang (2003)], in the more general case of cellular contractions of fibroblasts on a compliant substrate.

In order to identify the intracellular locations where stress levels may be of sufficient magnitude to elicit a biochemical response, one needs to look more closely at the individual proteins or protein complexes that transmit the forces throughout the cell and their interconnections. Perhaps the most complete analysis of protein structures that connect focal adhesions, especially transmembrane integrins to the cytoskeleton via a collection of integrin-CSK linking proteins, is that compiled by Zamir and Geiger [Zamir and Geiger (2001)]. The mapping of protein-protein interactions is daunting, however, illustrating the multiple binding partners of each of the over 40 proteins associated with a focal adhesion complex. This picture simplifies somewhat if we seek to identify those specific sequences of linked proteins that might provide a pathway for force transmission from the integrins to the cytoskeleton. Several of these pathways are illustrated in Fig. 2, and these constitute a potential list of candidates for conformational change under the application of forces transmitted via the integrin receptors. While the distribution of forces and the pathway for force transmission will differ depending upon the nature of the applied force, the molecular receptors that constitute the transmembrane linkage, and the concentration and type of proteins associated with the adhesion complex, one possible pathway allows force to be transmitted via the transmembrane integrin receptor, focal adhesion kinase (FAK), talin, and vinculin ultimately reaching the actin cytoskeleton. Several potential sites of signaling can be identified along this pathway, for example, conformational changes in FAK, that might influence its affinity for one of several known binding partners, or perhaps more importantly, its enzymatic activity, leading to activation of various signaling molecules including Rho and Rac [see recent review Katsumi, Orr, Tzima, and Schwartz (2004)].

Consider one example pathway from Fig. 2. It can be seen that integrins bind to talin, which, in turn, connects with F-actin either directly or via vinculin. Talin and integrin also bind to FAK. This force pathway alone gives rise to several possibilities for mechanotransduction. It has been shown that stresses applied to existing focal adhesions lead to enhanced localization of FAK and one of its binding partners, paxillin [Sawada and Sheetz (2002)]. This could occur, for example, subsequent to a conformational change in the integrin receptor itself or in talin, if such a change were to increase the binding affinity of either to FAK. Paxillin localization could then occur either as a direct result of FAK localization, or might possibly be augmented by a force-induced conformational change in FAK since once bound to the focal adhesion, it too becomes part of the force transmission pathway. All this leads to an accumulation of proteins to the focal adhesion complex, and a functional strengthening of the CSK-integrin attachment. But this does not directly explain the initiation of a signaling cascade. FAK, however, is known to possess several sites of tyrosine phosphorylation, one of which (Tyr-397) creates a binding site for the SH2-domain found in various focal adhesion proteins, and which has been shown to initiate signaling via the ERK1/2 pathway [Katz, Teng, Thomas, and Landesberg (2002)]. Thus, this one example demonstrates two types of mechanotransduction, both arising from protein conformational change, one leading to a strengthening of cell-matrix attachments, the other initiating an intracellular signaling cascade with numerous downstream implications.

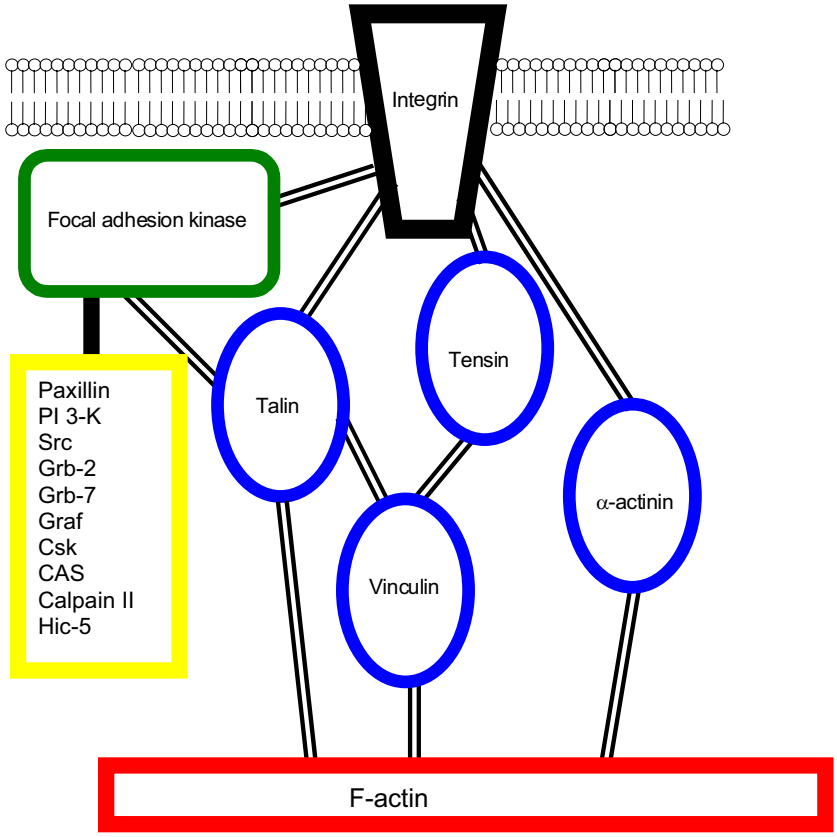

Figure 2 : Schematic representation of some of the proteins involved in transmitting force through a focal adhesion complex. Forces are transmitted across the cell membrane through the integrin receptors, then through a series of focal adhesion proteins that ultimately connect with the F-actin cytoskeleton. Conformational changes in any of these proteins might cause strengthening of the focal adhesion or initiate intracellular signaling. The known binding partners of focal adhesion kinase are also shown, many of which have been implicated in the process of mechanotransduction.

4 Force levels that initiate a cellular response

Recent advances in the use of atomic force microscopy and optical traps for single molecule manipulation have shed considerable light on the levels of force needed either to extend a protein or to rupture a protein-protein bond. For example, strong bonds such as those between integrins and several RGD-containing ligands have been measured to be between 30 and 100 pN [Lehenkari and Horton (1999)] and bonds between streptavidin and biotin rupture at forces in the range of 5 to 170 pN depending on the rate of force application [Merkel, Nassoy, Leung, Ritchie, and Evans (1999)]. Using atomic force microscopy, repeated domain unfolding has been demonstrated in several proteins, titin for example, under forces in the range of 80-200 pN [Oberhauser, Hansma, Carrion-Vazquez, and Fernandez (2001)], while talin 1 was found to form a molecular slip bond that ruptured under forces as low as 2 pN [Giannone, Jiang, Sutton, Critchley, and Sheetz (2003)]. Conformational changes that accompany protein stretching are expected to occur at lower forces, likely in the range of 10's of pN [Bao (2002)], although direct support for this estimate, either experimental or computational, is difficult to identify. Such conformational changes can involve displacements on the order of 1-5 nm, so the corresponding energy change, as indicated by the work done on the protein to produce the conformational change, would fall in the range of 10-100 pN·nm. Finally, if the cell is to use conformational change as a reliable means of initiating a force-induced response, the extended conformation should be attained only rarely in the absence of

force. Consequently the energy barrier to conformational change should be large compared to thermal excitation of kT $\sim$ 4 pN·nm if it is to be useful as a means of force sensation by the cell.

Geiger and co-workers [Geiger, Bershadsky, Pankov, and Yamada (2001)] and others have recently suggested that cells use active contractile force as a means of controlling cell function, maintaining tension in focal adhesions close to the threshold for signaling. Consistent with this hypothesis, measured values for the force exerted by a focal adhesion are approximately 5.5 nN/μm^2 [Balaban, Schwarz, Riveline, Goichberg, Tzur, Sabanay, Mahalu, Safran, Bershadsky, Addadi, and Geiger (2001)], leading to estimates for the force acting on a single integrin receptor of several pN, assuming close packing in the membrane. Viewed from a different perspective, forces of about 1 nN applied to the apical surface of a cell by magnetic beads have been found to elicit a response from adherent endothelial cells [Mack, Kaazempur-Mofrad, Karcher, Lee, and Kamm (2004)], and this is approximately equal to the total force exerted on an endothelial the cell by a 1 Pa shear stress, the approximate threshold for stimulation by hemodynamic shear [Davies (1995)].

5 Direct evidence for mechanotransduction as a consequence of protein conformational change

Little evidence can be found, that establishes a direct link between force-induced conformational change and the signaling cascades initiated by mechanotransduction. Even in the case of stretch-activated ion channels such as the MscL, for which the crystal structure is known [Chang, Spencer, Lee, Barclay, and Rees (1998)], the precise mechanism by which ion conductance is changed by membrane stress remains elusive. Recent molecular dynamics simulations show an increase in minimum pore diameter, from about 2 to 5 angstroms under physiologic levels of membrane stress [Gullingsrud, Kosztin, and Schulten (2001)]. This might represent an initial, tension-sensitive transition subsequently leading to the formation of a larger $\sim$30 angstrom pore as is necessary to explain the observed changes in conductance [Sukharev, Sigurdson, Kung, and sachs (1999)], but this remains to be demonstrated. In another example, the mechanism by which the ion channels located in the stereocilia of a hair cell are activated remains a source of debate [see Hamill and Martinac (2001)].

While single molecule experiments hold considerable promise for exploring these questions, little progress has been made to date. As discussed above, numerous experiments have demonstrated that single bond rupture events can be identified and their force levels measured. Progressive domain unraveling in proteins with repeating structures has also been demonstrated for proteins such as titin [Rief, Gautel, Oesterhelt, Fernandez, and Gaub (1997)] [Kellermayer, Smith, Granzier, and Bustamante (1997)], spectrin [Law, Carl, Harper, Dalhaimer, Speicher, and Discher (2003)] , and fibronectin [Oberhouser, Badilla-Fernandez, Carrion-Vazquez, and Fernandez (2002)]. In the case of fibronectin, these experiments provide some indirect evidence in support of the hypothesis that conformational change can alter binding characteristics, in that the domain disruption is believed to expose cryptic binding sites that would presumably become active once the protein is stretched [Hocking, Smith, and McKeown-Longo (1996)] [Zhong, Chrzanowska-Wodnicka, Brown, Shaub, Belkin, and Burridge (1998)]. Yet, the critical experiment providing definitive evidence that binding interactions can be induced by the application of force to a single protein has not yet been reported.

6 Force-induced changes in the focal adhesion-targeting domain of focal adhesion kinase.

As an important protein within the signaling pathway, FAK is suitable for testing the hypothesis that force-induced conformational changes in proteins play a critical role in controlling cell signaling pathways. Of particular interest in this regard is the focal adhesion targeting (FAT) domain of FAK that besides localizing to focal adhesions also binds paxillin. Binding of paxillin to FAT results in phosphorylation of paxillin [Cooley, Broome, Ohngemach, Romer, and Schaller (2000)] and through this binding, it is therefore one of the proteins that provides a potential link between the integrins in the cell membrane and the cytoskeleton [Geiger and Bershadsky (2002)].

The $\sim$150-residue region termed FAT resides near the C-terminal end of FAK, and is organized into four α-helix bundles of dimensions 6x2x2 nm, with the helices straight, closely antiparallel and connected by short ordered turns (see Figure 3). The bundle is highly compact and symmetrical with a square cross-section and a hydrophobic core known to be highly conserved across all species [Hayashi, Vuori, and Liddington (2002)]. On two

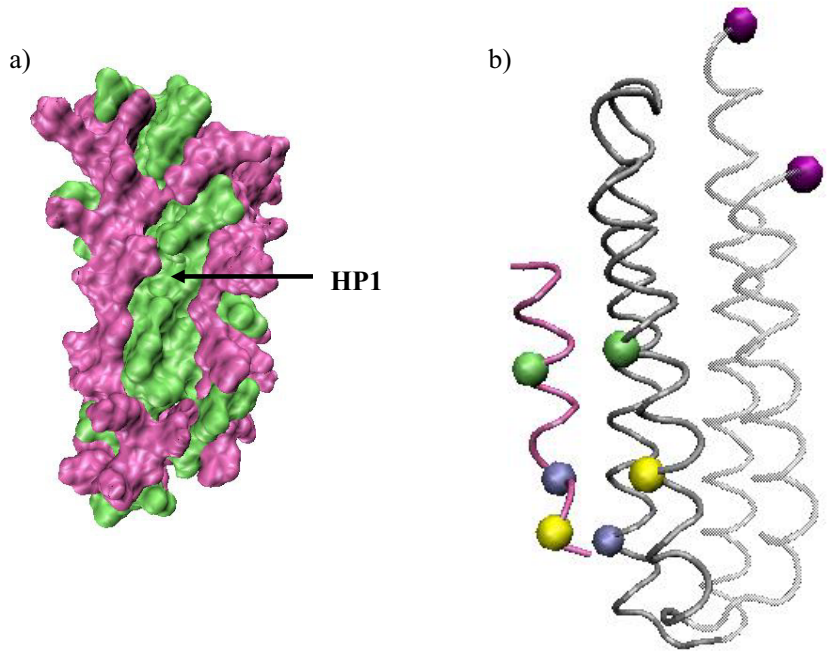

Figure 3 : (a) Surface hydrophobicity profile of FAT molecule. (b) Cartoon representation of FAT-Paxillin complex. The termini C-α atoms are shown as magenta Van der Waals spheres while the green, blue and yellow spheres indicate the pairing between LD C-α atoms and FAT C-α atoms that form salt bridges between the motifs and which are tracked throughout the unraveling of FAT-Paxillin throughout these simulations.

of its surfaces are exposed hydrophobic patches, HP1 and HP2, located on the faces of helix2-helix3 and helix1-helix4, respectively (see Figure 3(a)).

Paxillin is a 559 amino acid adapter protein with an apparent molecular weight of 68kDa. [Tumbarello, Brown, and Turner (2002)]. Within its N-terminal region are five distinct leucine-rich LD motifs, two of which have been found to be equally capable of binding to either HP1 or HP2.

We utilized steered molecular dynamics (SMD) simulation techniques to monitor how changes in the molecular conformation of FAT, induced by direct application of force, would affect its binding affinity to paxillin. Below, we introduce the simulation methods and provide a brief overview of our results on the force-induced, mechanical unfolding of FAT and the corresponding effect on FAT binding with paxillin. The reader is referred to a companion paper [Kaazempur-Mofrad, Golji, Abdul Rahim, and Kamm (2004)] for further details on the methods and implications of this study.

6.1 Steered Molecular Dynamics Simulations

The crystal structure of the complex of FAT bound with the LD motif of paxillin as determined by Hayashi et al. [Hayashi, Vuori, and Liddington (2002)] was obtained from the Protein Data Bank. This complex involves a theoretical model with hypothetical LD motif peptides. A commercially available molecular dynamics program CHARMM (version c27b) was utilized, and programmed to allow for constant force application on the protein molecule. The analytical continuum electrostatics (ACE) model was used to incorporate the solvent effects by approximating the solvent as a dielectric continuum where both the electrostatic as well as non-polar (non-electrostatic) solvation free energies contribute to the effective (free) energy. With temperature maintained at 310K, the N-terminal C-α atom was fixed and a constant force was applied to the C-terminal C-α atom, along a vector pointing away from the N-terminal C-α atom. Due to lack of data on the actual, physiological configuration of force application on FAT, this end-to-end pulling direction was used as an arbitrary configuration for perturbing the system in order to qualitatively un-

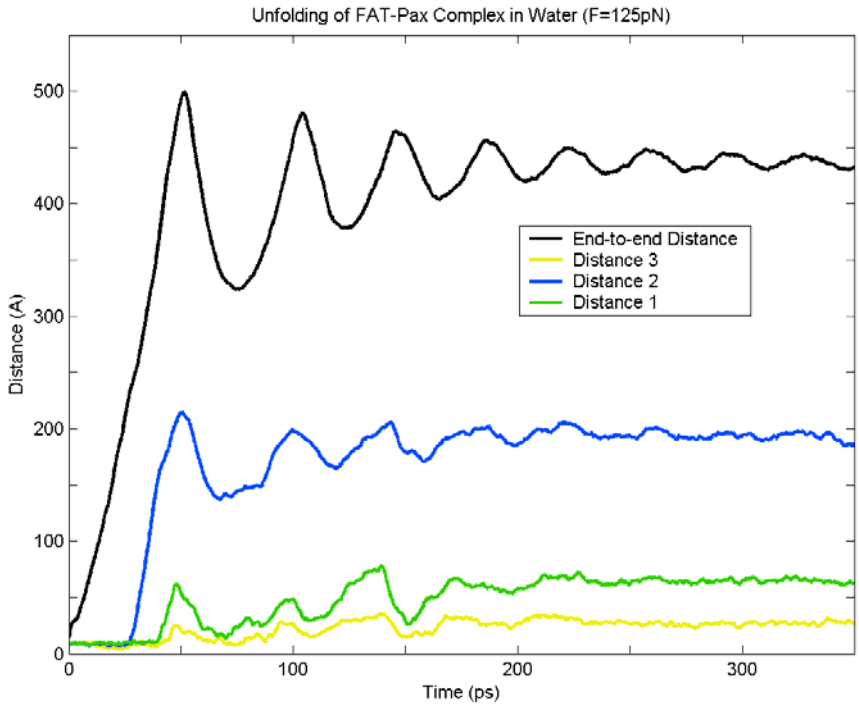

Figure 4 : Mechanical unfolding of FAT-Paxillin complex with a 125pN mechanical force. The FAT terminal end-to-end distance is shown by the black line and the three C-α atom pair distances are matched with their Van der Waals pairing colors from Figure 3.

derstand its mechanics. SMD simulations were carried out on the FAT complex with LD motif of paxillin (FAT-Pax) at different constant values of applied force until unfolding occurred. Levels of applied force were chosen so as to unravel the FAT molecule within an acceptable timeframe $\sim$10ns. The analysis of MD trajectories was conducted using a visualization software package, VMD. The change in end-to-end distance between the two termini backbone carbon atoms of FAT was used as an indicator of progress of the unfolding process.

We explored the unfolding pathways of FAT bound to the LD motif of paxillin, and examined how the force-induced conformational changes in FAT would influence its binding affinity to paxillin. Specifically, the terminal end-to-end evolution with force application was monitored, as well as the binding affinity between FAT and paxillin. Binding affinity is characterized here by the distance between three pairs of C-α atoms on FAT and the LD motif of paxillin, respectively, that form salt bridges along the length of the hydrophobic groove HP1 (see Figure 3, where the C-α atoms are sketched in spherical Van der Waals representation and the corresponding distances are termed Distance 1, Distance 2 and Distance 3, respectively).

Two levels of constant force were applied, namely 75pN and 125pN. A 75pN force was not sufficient to unfold the complex in 10ns, the maximum length of the SMD simulation (data not shown). At 125pN, however, FAT unraveled smoothly without any obvious stable intermediary structures before complete unfolding was achieved at 50ps (see Figure 4). Increased force levels reduced the unfolding time; e.g., only 45ps was needed with a 150pN force. Unfolding of the FAT molecule was immediately followed by the unbinding and release of paxillin from the complex.

These results demonstrate that the integrity of FAT, especially the conformation of the hydrophobic groove HP1, is crucial for binding of paxillin to FAT. With the distortion of this hydrophobic patch, the LD motif of paxillin is unable to align with the hydrophobic groove HP1 and FAT-paxillin binding can not occur. The SMD simulations show that HP1 is kept intact and is the last to unravel, indicating that formation of the FAT 4-helix bundle through alignment of its particular set of amino acids is

optimized for promoting the paxillin binding event (see [Kaazempur-Mofrad, Golji, Abdul Rahim, and Kamm (2004)] for further detail).

The present example demonstrates, though indirectly, that force-induced conformational change in individual proteins is a likely mechanism for transduction of mechanical signals carried via alteration of binding events in the mechanosensing pathways.

7 A look ahead

Progress is rapidly being made on both the experimental and computational fronts, which will likely lead soon to a direct demonstration of force-induced changes in binding affinity. Using either AFM or optical tweezers, controlled force applications in the pN range and displacement measures in the nm range are well within current capabilities. At the same time, single-molecule fluorescence measurements provide the opportunity to monitor single binding events. On the computational side, several barriers exist, but these are also being surmounted, although slowly. Steered molecular dynamics can be applied to proteins to predict their conformational change under force, and docking simulations provide a means for determining binding affinities in different conformational states. The barriers to progress lie primarily in our lack of atomistic models with adequate resolution for those proteins of greatest interest, located in the force transmission pathway. These tend to be difficult to crystallize so few structures are available. Moreover, due to their size, simulations are computationally intensive, especially if the presence of water molecules is included explicitly. Despite these constraints, some progress can be made by using subdomains of the proteins of interest, provided their functionality can be demonstrated.

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