FINAL REPORT

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TITLE: Mechanotransduction through Cytoskeleton

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The goal of this project was to characterize the molecular mechanism by which cells recognize and respond to physical forces in their local environment. The project was based on the working hypothesis that cells sense mechanical stresses, such as those due to gravity, through their cell surface adhesion receptors (e.g., integrins) and that they respond as a result of structural arrangements with their internal cytoskeleton (CSK) which are orchestrated through use of tensegrity architecture. In this project, we carried out studies to define the architectural and molecular basis of cellular mechanotransduction. Our major goal was to define the molecular pathway that mediates mechanical force transfer between integrins and the CSK and to determine how mechanical deformation of integrin-CSK linkages is transduced into a biochemical response. Elucidation of the mechanism by which cells sense mechanical stresses through integrins and translate them into a biochemical response should help us to understand the molecular basis of the cellular response to gravity as well as many other forms of mechanosensation and tissue regulation.

The specific aims of this proposal were:

1. To define the molecular basis of mechanical coupling between integrins, vinculin, and the actin CSK
2. To develop a computer simulation of how mechanical stresses alter CSK structure and test this model in living cells
3. To determine how mechanical deformation of integrin-CSK linkages is transduced into a biochemical response.

Accomplishments:

Specific Aim 1:

We studied vinculin-deficient (Vin -/-) F9 embryonic carcinoma cells that spread more slowly and to a lesser degree on fibronectin (FN)-coated dishes compared to wild type (Wt) cells. These experiments revealed that decreased cell spreading was accompanied by inhibition of lamellipodia extension, reduced stress fiber formation, and

decreased mechanical coupling between integrins and the internal CSK, as determined using cell magnetometry and atomic force microscopy. Furthermore, when vinculin was transfected back into the Vin-/- cell line, all these features were restored to near wild type level.

We then studied another line of vinculin-deficient F9 embryonic carcinoma (γ-229) cells that failed to spread or maintain effective CSK stiffness relative to wild-type F9 cells, as detected using atomic force microscopy. Transfection of γ-229 cells with the head domain of vinculin lacking the hinge region (amino acids 1-850) or the tail domain (858-1066) were unable to reverse these effects. While simultaneous expression of the head and tail domains was slightly more effective, it did not completely restore normal cell shape and mechanics as did replacement with intact vinculin. In contrast, deletion of the vinculin's tyrosine phosphorylation site by substitution of tyrosine 822 with phenylalanine did not interfere with normal cell function. Similar effects was observed in studies on control of lamellipodium formation: microinjection of constitutively-active rac only induced extension of lamellipodia in cells that expressed intact vinculin protein and again, deletion of the tyrosine phosphorylation site was not required for this activity. These results demonstrate that the vinculin's ability to mechanically couple integrins to the CSK, to modulate cell mechanics, and to promote cell shape changes depends on its three dimensional structure, in addition to its protein-protein binding functions. Importantly, mechanical coupling between intact vinculin, integrins, and other CSK proteins in the focal adhesion also appears to be required for activated rac to manifest its ability to promote lamellipodium formation and hence, drive cell motility.

In a separate study, a new magnetic tweezer apparatus was constructed to apply controlled tensional forces (10 pN to greater than 1 nN) to transmembrane receptors via bound ligand-coated microbeads while optically measuring lateral bead displacements within individual cells. Use of this system with wild-type F9 embryonic carcinoma cells and cells from a vinculin knockout mouse F9 Vin (-/-) revealed much larger differences in the stiffness of the transmembrane integrin linkages to the CSK than previously reported using related techniques that measured average mechanical properties of large cell populations. The mechanical properties measured varied widely among cells, exhibiting an approximately log-normal distribution. The median lateral bead displacement was 2-fold larger in F9 Vin (-/-) cells compared to wild-type cells whereas the arithmetic mean displacement only increased by 37%. We conclude that vinculin serves a greater mechanical role in cells than previously reported and that this magnetic tweezer device may be useful for probing the molecular basis of cell mechanics within single cells.

Specific Aim 2:

This aim was based on our working hypothesis that cells use tensegrity architecture to control their shape and mechanical stability. This class of structures maintains shape stability within a continuous, tensed network of structural members by incorporating other isolated support elements that resist compression. The deformability of these structures depends on the level of prestress (pre-existing tension) in the structure before application of an external load. In the simplest embodiment of the cellular tensegrity model, this stabilizing prestress is generated actively by the cell's

actomyosin-based contractile apparatus and passively by distension through the cell's adhesions to extracellular matrix (ECM). The model assumes that the prestress is carried primarily by tensile microfilaments and intermediate filaments. This prestress is balanced by interconnected structural elements that resist being compressed, including internal microtubules, and by traction on the ECM. Thus, this model differs from the established continuum models in that it leads to predictions relating to the mechanical role of distinct molecular elements and it suggests a central unifying role for prestress.

In a past funding period, we developed a mathematical formulation of this theory starting from first principles. During this funding period, this model was greatly extended and refined by our collaborator, Dr. Dimitrije Stamenovic (Dept. of Biomedical Engineering, Boston U.). Importantly, we have demonstrated that the model has qualitative and quantitative consistencies with experimental results in various cell types. Nevertheless, tensegrity remained controversial because key pieces of evidence that are essential for its validation were missing. These critical experiments included unequivocal demonstration that the CSK behaves as a discrete network composed of different types of CSK filaments; direct evidence that microtubules function as compression struts and contribute significantly to cell mechanics; quantitative measurements demonstrating that CSK prestress is a major determinant of cell deformability; and experimental confirmation of *a priori* predictions of the theoretical tensegrity model.

During this funding period, we used real-time microscopic analysis of cells containing GFP-labelled microtubules and associated mitochondria to show that living cells behave like discrete structures composed of an interconnected network of actin microfilaments and microtubules when mechanical stresses are applied to cell surface integrin receptors. Quantitation of cell tractional forces and cellular prestress using traction force microscopy confirmed that microtubules bear compression and are responsible for a significant portion of the cytoskeletal prestress which determines cell shape stability under conditions in which myosin light chain phosphorylation and intracellular calcium remained unchanged. Quantitative measurements of both static and dynamic mechanical behaviors in cells also were consistent with specific *a priori* predictions of the tensegrity model. These findings suggest that tensegrity represents a unified model of cell mechanics which may help to explain how mechanical behaviors emerge through collective interactions among different cytoskeletal filaments and extracellular adhesions in living cells.

Interestingly, this new view of cell structure has led to novel insights into how life may have first originated on planet earth. The thesis is that the same architectural and energetic constraints that shape cells today also guided the evolution of the first cells and that the molecular scaffolds which support solid-phase biochemistry in modern cells represent living microfossils of past life forms. This concept emerged from the discovery that cells mechanically stabilize themselves using tensegrity architecture and that these same building rules guide hierarchical self-assembly at all size scales. When combined with other fundamental design principles (e.g., energy minimization, topological constraints, structural hierarchies, autocatalytic sets, solid-state biochemistry), tensegrity provides a physical basis to explain how atomic and molecular elements progressively self-assembled to create hierarchical structures with increasingly complex functions, including living cells that can self-reproduce. This work

implies that similar architectural rules may guide how life originates on other planets as well.

<u>Specific Aim 3</u>:

To dissect the molecular basis of transmembrane mechanochemical signaling, controlled shear stresses were applied directly to surface receptors on living cells by magnetically twisting bound ligand-coated microbeads. Twisting integrin receptors resulted in stress-dependent increases in Gsα activation, intracellular cyclic AMP, nuclear translocation of the catalytic subunit of protein kinase A (PKA), phosphorylation of the transcriptional regulator CREB, and transcription of a gene reporter driven by the cAMP response element. In contrast, mechanical force application through a metabolic transmembrane receptor failed to activate these signaling events. Stress-dependent signaling through the cAMP pathway was completely prevented by inhibiting activation of heterotrimeric Gα proteins using GDP-β-S whereas disruption of connections between integrins and the internal CSK had no effect. These results demonstrate that transmembrane integrin receptors mediate mechanochemical transduction by activating heterotrimeric G proteins locally with the focal adhesion at the site of integrin binding and thereby triggering an intracellular cAMP signaling cascade that leads to changes in gene transcription. More generally, this work is important because it has led to discovery of the key role that integrins play in mechanotransduction in all adherent cell types and hence, their potential role in gravity sensation.

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