31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

The Foldome in Cellular Force Transduction

Dennis E. Discher

Abstract— **The Human Genome is essentially complete, and yet the impact on how we understand physiological processes such as cellular force transduction has been minimal in part because of our inability to work from known sequence to structure, i.e. the Foldome. In order to specifically identify cytoskeletal proteins that change conformation or assembly in stressed versus static cells, in situ labeling of sterically-shielded or 'cryptic' cysteines with fluorophores is analyzed by quantitative mass spectrometry, sequential two-dye labeling, and fluorescence imaging. Within red blood cells, shotgun labeling shows that shielded cysteines in the two isoforms of the cytoskeletal protein spectrin are increasingly labeled as a function of shear stress and time, indicative of forced unfolding of specific domains. Within mesenchymal stem cells – as a prototypical nucleated cell – non-muscle myosin IIA and vimentin are just two of the cytoskeletal proteins identified that show differential labeling in tensed versus drug-relaxed cells. Cysteine labeling of proteins within live cells can thus be used to fluorescently map out sites of molecular-scale deformation, and the results also suggest means to co-localize signaling events such as phosphorylation with forced unfolding.**

I. Introduction

Force-induced changes in protein conformation have long been postulated to be determinants of cell deformability (1). Likewise in adhesion, forces of pico-Newton magnitude that result from cells pulling on matrix are believed to induce conformational changes that initiate essential anchorage signals. Single molecule measurements indeed show that domain unfolding occurs in reversible extension of purified cytoskeletal, motor, and matrix adhesion proteins, and molecular dynamics simulations of protein extension have clarified mechanisms. Direct cell-level evidence is lacking or even contrary to forced unfolding, although cytoskeletal binding of a large and rare conformation-sensitive antibody has suggested extension of a proline-rich region in one protein within spread, fixed cells. In a broad 'shotgun' approach here using small thiol-reactive probes and live cells under physiological stresses, differential labeling of force-exposed cysteines directly demonstrates in-cell structural changes of specific domains in multiple cytoskeletal proteins (2).

Cysteine (Cys) is a moderately hydrophobic amino acid that it is frequently shielded by tertiary or quaternary protein structure. Labeling of cysteine's SH moiety has been exploited in solution denaturation studies on a few small purified proteins as well as in an anemia-causing proline mutation in the red blood cell protein spectrin. In addition, forced unfolding of single proteins with core-sequestered disulfides demonstrate reduction of the S-S within seconds by reactive thiols in the medium. We show here in intact cells under physiological conditions that stress-associated changes in protein structure can also expose – for relatively rapid reaction – specific buried Cys in a number of key cytoskeletal proteins. Sequential *n*-dye labeling using different colors ($n = 2$ here) proves to be a useful approach to amplifying signals from cryptic sites relative to pre-labeled surface sites. We illustrate the range of this in situ 'Cys shotgun' approach first with the relatively simple human red blood cell that allows for the most direct demonstration of forced unfolding in fluid-stressed cells and then with cardiomyocytes and human mesenchymal stem cells under cell-generated tension.

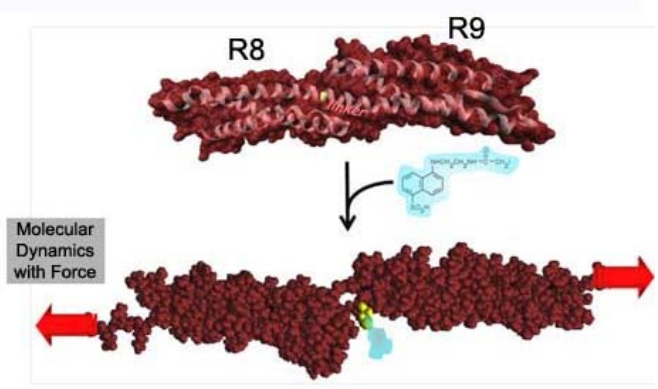

Fig. 1. In the one published erythroid Spectrin Repeat structure, β-Spectrin's Cys^{1167} is sequestered (0 Å exposed). Forced extension *in silico* exposes the SH group for reaction.

Red blood cells (RBC) deform under the incessant stresses of blood flow, and the spectrin membrane cytoskeleton has proven central to cell deformability. Spectrin's a and b chains interact to crosslink F-actin in this cell, and in single molecule studies spectrin's tandem array of helical bundle domains are found to unfold at low forces (~picoNewton) that could be generated by just a few myosin motor molecules. There are 20 Cys in a-spectrin and 15 Cys in b-spectrin, and some of these are buried in crystal structures and homology models. To assess exposure of Cys in unfolding of spectrin and all of the other RBC membrane proteins, cells were reversibly lysed to make hemoglobin-depleted pink 'ghosts' that were resealed with entrapment of a Cys reactive fluorophore. Dye-loaded cells were then either held static in suspension at various temperatures or else sheared over a physiological range of stress using a standard fluid shearing device. After 5 min or more, cells

Manuscript received April 7, 2009.

D.E. Discher and coworkers are with the University of Pennsylvania: 215-898-4809; (e-mail: discher@seas.upenn.edu).

were re-lysed, excess dye was quenched, and cells were imaged to assess membrane labeling.

Solubilized cells were denatured and all Cys that were not dye labeled were alkylated. Separation of membrane proteins by 1D SDS-PAGE followed by densitometry showed 50% higher dye fluorescence in the bands of α and β spectrin from the shear samples (15 runs at 37°C); sequential two-dye labeling magnifies this difference to >500%, as described below. Labeling under shear is only enhanced for spectrin: labeling of the other major membrane proteins (ankyrin, protein 4.1, actin, etc.) was not affected by fluid shear, which suggests together with additional results below that mixing is not limiting.

Liquid chromatography coupled tandem MS (LC-MS/MS) was used to identify and quantify Cys-dye modified sites in spectrin bands after excision and trypsinization. Within a- and b-spectrin, respectively, 13 and 14 Cys-dye adducts were detected. Since surface-exposed Cys are predominantly dye labeled and buried Cys are largely inaccessible until subsequent denaturation and aceylation, site-specific ratios $f(t)$ (= shear/static) of dye labeling in ion chromatogram elution profiles quantify Cys exposure. A majority of ratios are the same for shear and static samples, but at least six Cys sites are distinct with results that are typically in the range of $f \approx$ 5-10 at 60 min but as high as 36. Cleavage patterns of spectrin treated with NTCB (2-nitro-5-thiocyanobenzoic acid), which cleaves peptide bonds at unlabeled Cys, are distinct for shear and static samples, consistent with qualitative differences in Cys exposure.

The structure of shear sensitive b-R8-9 shows that the shear-labeled Cys^{b1167} is 100% buried or shielded within a tertiary fold – at least until force induces localized unfolding. A recombinant multi-domain construct β-R5-9 was therefore Cys-labeled at different temperatures for a fixed time and then analyzed by both fluorescence and MS. Construct labeling occurs only at $T \geq 25$ °C, with evidence from MS of Cys^{b1167} labeling at 25°C. This apparent transition corresponds to the T_m of b-R9 and correlates with both the thermally induced loss of helicity and the loss of mechanical stability of R9 as determined by AFM forced extension. At higher temperatures, additional repeats are labeled as helicity is lost.

The force-sensitive Cys^{a1203} was also studied in solution with a recombinant a-R12 domain for which modeling predicts partial shielding. At a given temperature, the ratio of dye labeling rates $F(T)$ for urea-denaturing conditions versus native conditions relates to the steric protection of a partially exposed Cys. The recombinant's $F(37°C) \approx 4$ approximates the LC-MS/MS result for shear versus static cells' $F(37\ °C) = 5$. Normalization and rescaling to the folded state maximum gives the Fraction Folded, and this decreases with T in parallel again to the helicity loss upon heating.

Based on the above detailed molecular understanding of labeling 'cryptic' Cys, an additional cellular system has been studued [3]. Embryonic cardiomyocytes cultured on a series of flexible substrates show that matrices which mimic the elasticity of the developing myocardial microenvironment are optimal for transmitting contractile work to the matrix and for promoting actomyosin striation and 1 Hz beating. On hard matrices that mechanically mimic a post-infarct fibrotic scar, cells over-strain themselves, lack striated myofibrils and stop beating; on very soft matrices, cells preserve contractile beating for days in culture but do very little work. Optimal matrix leads to a strain match between cell and matrix and suggests dynamic differences in intracellular protein structures. The "Cysteine Shotgun" method of labeling the *in situ* proteome reveals differences in assembly or conformation of several abundant cytoskeletal proteins, including vimentin, filamin, and myosin. The results highlight the need for greater attention to fibrosis and mechanical microenvironments.

CONCLUSION

Changes in the Foldome within mechanically active or stressed cells should lend deeper insight into cellular force transduction.

REFERENCES

[1] E. A. Evans, R. Skalak, *Mechanics and Thermodynamics of Biomembranes*. (*CRC Press.* Boca Raton, 1980), p190.

[2] C.P. Johnson, H-Y. Tang, C. Carag, D.W. Speicher, and D.E. Discher. Forced unfolding of proteins within cells. *Science* 317: 663-666 (2007).

[3] A. Engler, C. Carag, C. Johnson, M. Raab, H-Y. Tang, D. Speicher, J. Sanger, J. Sanger, and D.E. Discher. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *Journal of Cell Science* 121: 3794-3802 (2008).