Interface Concentration Dependence in Unfolding Proteins using AFM

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ABSTRACT

Filamin A is a long, high molecular mass (~280 kDa) acting-binding protein, constituting of 24 Ig domains in a monomeric form. Filamin A cross-links F-actin into three-dimensional isotropic resilient, stiffer, more solid-like networks. Dysfunction or deletion of human filamin causes various genetic disorder or deformations in melanoma cells due to reduced stiffness of cortical actin. Filamin's key role in actin organization, membrane stabilization, and the anchoring of transmembrane cell receptor proteins to the actin cytoskeleton, suggests that filamin has an important mechanical function. Here we show by atomic force microscopy (AFM) that all repeats for filamin extend and unfold at similar forces as observed and analyzed through sawtooth patterns. This novel AFM based nano-biotechnology opens a molecular mechanic approach for studying structure to function related properties of any type of individual biological macromolecules. In order to study forced unfolding of sub-domains within filamin, the minimal possible forces to unfold native filamin must be determined by eliminating effects of multiple chains or loops as much as possible. Here we show that force for unfolding individual domains of intact filamin increase with the increase in protein concentration adsorbed on the substrate (mica/gold) and thus results into high range of forces for unfolding as reported in previous studies. Similar interfacial concentrations dependent AFM induced unfolding experiments were performed and verified on titin-$(I27)_8$ peptide. Further domain unfolding forces for full length filamin monomer were compared with its sub-domain constructs- $(R15\text{-}R16)_m$ – consisting of repeats 15 and 16.

INTRODUCTION & RESULTS

Filamin-A, a 280 kDa actin binding protein (also known as ABP-280) with 24 Ig domains is studied with the aim of understanding the effects of protein concentration on unfolding forces in AFM extension. Of additional interest, filamin has a long flexible proline rich linker region between domains 15 and 16 which can be further studied to assess its role in extensibility and perhaps co-operative unfolding.

Filamins are about as long as another actin binding protein, dystrophin, which has 24 spectrin repeats instead of 24 Ig domains. While both proteins are found in muscle and many other tissues, filamins are

unlike dystrophin in that they can homodimerize at the C-terminal domain. As a major component of microfilament networks, filamins have major effect on the rheology of cross-linked networks in vivo and in vitro (3-6). Filamin-A also interacts with integral membrane proteins integrin -β1 and -β2 (7,8). Dysfunction or deletion of human filamin causes various genetic disorder or deformations in melanoma cells due to reduced stiffness of cortical actin (9,10). Most of this protein contains stretches of anti-parallel β-sheet conformation in the Ig-fold (11,12).

To explore the forced unfolding pathways of a single molecule of filamin-A monomer, molecule is stretched in aqueous solution using AFM. The characteristic sawtooth patterns of unfolding appear similar to those reported for titin, spectrin and dystrophin. Full analyses of the thousands of force spectrograms is done by first categorizing each according to the number of peaks as well as the sawtooth patterns' peak-to-peak lengths and force amplitudes. Further an extended concentration dependent forced unfolding study on filamin-A monomer is performed. Earlier work on filamin-A by Furuike et. al. (13) reported the force distribution for the unfolding of filamin domains was trimodal and ranged from 50 to 220 pN, with unfolding intervals sharply distributed around 32 nm. Furuike et. al. (13) further argued that this multi-modal distribution of unfolding forces can be explained by variation in values of activation energy and the width of activation barrier of 24 Ig-fold domains of the filamin-A at the unfolding transition, thus proposing that all the 24 domains in filamin-A molecule are not identical.

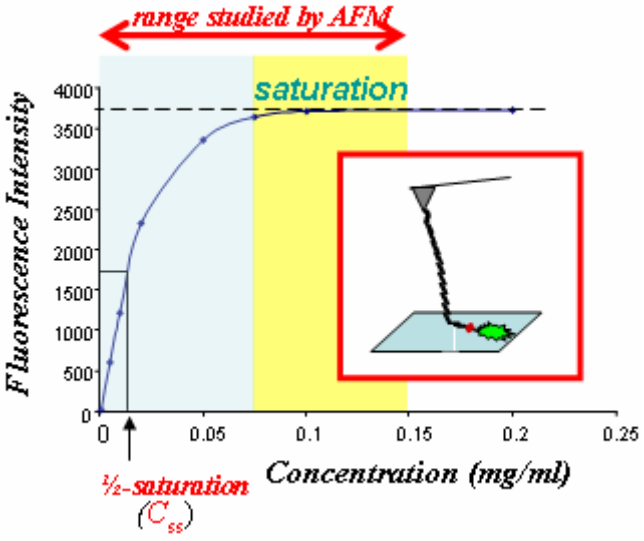


Figure 1: *Adsorption studies for GFP-Filamin-A monomer. Protein was adsorbed for 15 mins to glass.*

In order to study forced unfolding of sub-domains within filamin, the minimal possible forces to unfold native filamin must be determined by eliminating effects of multiple chains or loops as much as possible. Effects of loop is also illustrated in case of spectrin, dystrophin etc by a second small Gaussian for domain unfolding force. To investigate filamin adsorption, we performed a fluorescence adsorption study on filamin-A monomer tagged with GFP at one end and chopping off one self-dimerizing domain at the other end. The intensity or adsorbed mass for various concentrations of the labeled proteins was measured using fluorescence microscopy. Figure 1 plots the adsorbed mass/intensity versus concentration and shows that saturation is reached by 0.075 mg/ml of filamin concentration. The graphs fit very well to the saturable Langmuir adsorption isotherm equation. As also described in previous studies we incline to work at halfway (C_{ss}) of the linear region (sub-saturation regime) in figure 1. In our AFM experiments various concentrations of protein were used in order to compare the unfolding force and length data with adsorption profile of protein on the mica.

The sawtooth patterns from AFM tip retractions recognized as unique to protein unfolding with forces ranging from 50 to 300 pN at intervals of 32 nm. One such experiment done at very high protein concentration (~ 3 x C_{ss}) generated similar force distribution histograms as shown by Furuike et. al (13) (Figure 2). Force distributions cumulated from the heights of the force peak are reported to be trimodal

with Gaussian means differing by about a factor of ~2 and ~3. The trimodal histograms were fitted with three Gaussians of the same width. The major Gaussian fit most likely reflects the force to unfold a single repeat, whereas the minor second and third Gaussian fits at twice and three times the force reflects the respective unfolding of two and three chains in parallel. To assess this hypothesis, AFM studies were performed after adsorbing varying protein concentrations onto the mica. The lowest concentrations gave fewer and fewer sawtooths and point to a need for alternative methods of identifying proteins on the surface for pulling.

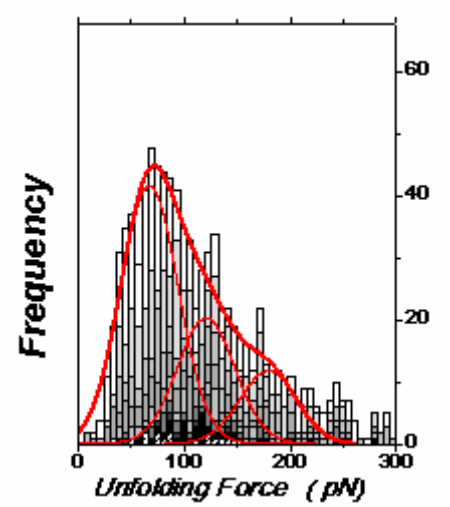
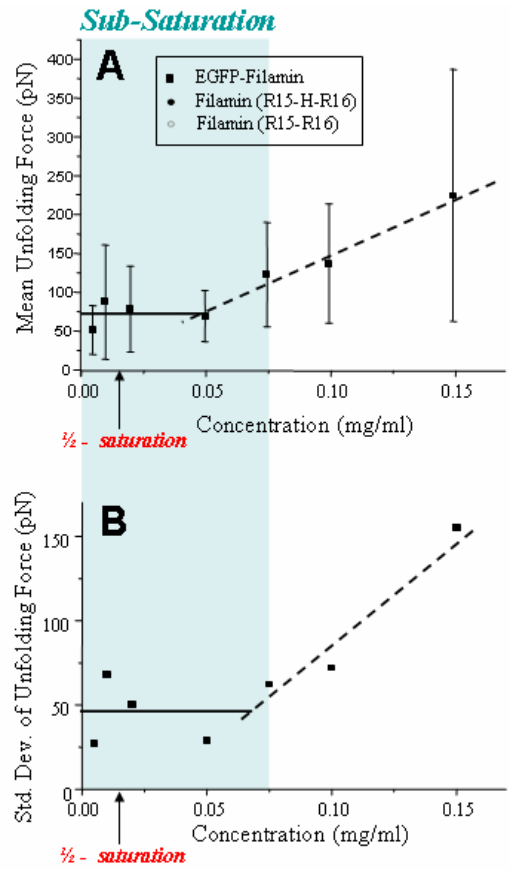


Figure 2: *Multi-modal unfolding force distribution for Filamin-A at high concentrations. Peak forces prove similar to Furuike et. al. (13).*

Plotting mean unfolding force, <F>, for different concentrations of protein adsorbed on to the substrate demonstrated a flat or saturated profile for sub-saturation regime (Figure 3A) as obtained by fluorescence imaging. Further increase in protein concentration results in linear increase of unfolding forces. Also the std. deviation for unfolding forces becomes larger for increasing protein concentration beyond sub-saturation (Figure 3B). For different concentrations of filamin adsorbed onto the substrate the heights of the force peaks are found to span a large range from 50 to 400 pN which is in accordance with Furuike et. al (13). These forces are clearly much higher than the 20-30 pN forces for spectrin (2), but similar to 150 – 200 pN as originally reported for titin under similar rates of protein extension (0.01–10 nm/msec) (1).



Unfolding length histograms here for filamin are more similar to titin than spectrin in a way that there are no or fewer instances of tandem event unfolding. We nonetheless conclude that the first major peak at ~ 32 nm in our $l_{pk\text{-}pk}$ histograms corresponds to the length gained in unfolding most of a single repeat which is similar to as reported by Furuike et. al. (13). Increase in adsorbed concentration of protein on the substrate does not affect peak-to-peak unfolding lengths. Therefore peak-to-peak unfolding length which is more of a characteristic of a protein being pulled remains unaffected of the surrounding environment.

From unfolding forces and lengths results using peak-to-peak histogram we establish that each domain in filamin molecule should unfold at a similar force with the extension of ~ 32 nm.

Figure 3: *Unfolding Force Analysis. (A) Dependence of average force for unfolding of Filamin A domain on concentration adsorbed on mica. (B) Plot of std. deviation of unfolding force versus concentration.*

Therefore typical sawtooth for a filamin molecule/sub-molecule should demonstrate peaks around 50 – 75 pN regular spaced at around 32 nm intervals. These results can further be used to study properties of any individual subdomain or its effect on surrounding domains. One such study we would like to propose to do in near future is to study the properties and effect of native hinge between repeat 15 and repeat 16 on unfolding of sub-domain R15-H-R16 and R15-R16. Comparison of unfolding results for sub-domain with or without hinge can be employed to establish hinge effects. Preliminary results on constructs R15-H-R16 and R15-R16 demonstrated low unfolding forces and lengths, with initial predictions for tandem or longer length unfolding in the case of R15-H-R16. Small forces and lengths for unfolding can be related to small size of the two constructs being studied. Moreover, with no previous AFM unfolding studies on such small β-sheet constructs, we propose to work on same constructs concatenated to themselves 2-4 times- $(R15\text{-}H\text{-}R16)_m$ and $(R15\text{-}R16)_m$ with m = 2-4.

Preliminary results on $(R15\text{-}H\text{-}R16)_3$ and $(R15\text{-}R16)_3$ show unfolding forces around 75 pN, which is similar to forces as demonstrated by filamin-A monomer. Further in order to evaluate concentration effects of shorter and less flexible construct proteins on unfolding forces, we performed AFM unfolding on titin-$(I27)_8$ that has already been extensively studied by Fernandez et. al.(1). Concentration dependent AFM studies similar to filamin-A monomer is performed on titin-$(I27)_8$. Unfolding length and force histograms compiled by analyzing fingerprint sawtooth patterns for unfolding predicts constant unfolding forces and lengths with increase in concentration. These results were reported to be very similar to one reported by Fernandez et. al.(1) with exceptions for std. deviation arising due to unbiased analysis of all the generated data. Initial guesses for unfolding peak-to-peak data depending on previous studies would estimate constant average unfolding length and increasing average forces with the concentration. Contrasting to the force estimate, average unfolding forces were observed to remain constant with the concentration. We hypothesize that in the case of shorter, less flexible chains it becomes rare to form interconnected loops at high concentration as is observed in longer filamin-A monomer. Concordance between our results for titin and by Fernandez et. al.(1) further displays approval of the analysis techniques that we employ in our AFM experiments.

REFERENCES

1. Reif, M., Gautel, M., Oesterhelt, F., Fernandez, J.M., and Gaub, H.E. 1997. Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM. *Science* **276,** 1109-1112.
2. Law, R., Carl, P., Harper, S., Dalhaimer, P., Speicher, D.W., and Discher, D.E. 2003. Cooperativity in forced unfolding of tandem spectrin repeats. *Biophys. J.* **84,** 533-544.
3. Hartwig, J., and Stossel, T. 1981. The structure of actin-bieding protein molecules in solution and interacting with actin filaments. *J. Mol. Biol.* **145**, 563-581.
4. Hartwig, J.H. and Shevlin, P. 1986. The architecture of actin filaments and the ultrastructural location of actin-binding protein in the periphery of lung macrophages. *J. Cell Biol.* **103**, 1007-1020.
5. Janmey, P.A., Hvidt, S., Lamb, J., and Stossel, T.P. 1990. Resemblance of actin-binding proteirdactin gels to covalently crosslinked networks. *Nature (Lond.).* **345**, 89-92.
6. Ito, T., Suzuki, A. and Stossel, T. 1992. Regulation of water flow by actin-binding protein-induced actin gelatin. *Biophys. J.* **61**, 1301-1305.
7. Sharma, C.P., Ezzell, R.M. and Arnaout, M.A. 1995. Direct interaction of filamin (ABP-280) with the beta 2-integrin subunit CD18. *J. Immunol.* **154**, 3461-3470.
8. Loo, D.T., Kanner, S.B., and Aru¡o, A. 1998. Filamin binds to the Cytoplasmic Domain of the β_1-Integrin. *J. Biol. Chem.* **273**, 23304-23312.
9. Cunningham, C.C., Gorlin, J.B., Kwiatkowski, D.J., Hartwig, J.H., Janmey, P.A., Byers, H.R., and Stossel, T.P. 1992 Actin-binding protein requirement for cortical stability and efficient locomotion.Science. **255**, 325-327.
10. Cunningham, C.C. 1995 Actin polymerization and intracellular solvent flow in cell surface blebbing. *J. Cell Biol.* **129**, 1589-1599.

11. Tyler, J.M., Anderson, J.M., and Branton, D. 1980. Structural comparison of several actin-binding molecules. *J. Cell Biol.* **85**, 489--495.
12. Koteliansky, V., Glukhova, M., Shirinsky, V., Smirnov, V., Bushueva, T., Filimonov, V., and Venyaminow, S. 1982. A structural study of filamin, a high-molecular-weight actin-binding protein from chicken gizzard. *Fur. J. Biochem.* **121**, 553-559.
13. Furuike, S., Ito, T., and Yamazaki M. 2001. Mechanical unfolding of single filamin A (ABP-280) molecules detected by atomic force microscopy. *FEBS Lett.* **498(1)**, 72-5.