Actin-binding Proteins in Physiology & Disease

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Structure Function Analysis of Disease-Causing Missense Mutations in Dystrophin

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Duchenne muscular dystrophy (DMD) affects 1 of every 3500 males and results in death during the mid to late twenties. Mutations in the dystrophin gene leading to DMD commonly result in loss of protein expression or expression of a truncated protein lacking essential ligand binding domains. In some cases, point mutations leading to a single amino acid change in the dystrophin protein cause DMD, Becker muscular dystrophy or X-linked cardiomyopathy. Of the known disease causing mutations, 9 are located in the N-terminal actin-binding domain of dystrophin. Examining the effects of these mutations on actin binding activity will lead to a better understanding of key residues for dystrophin function in vivo. With this in mind, we engineered all 9 N-terminal disease-causing mutations into the full-length dystrophin cDNA and have begun to characterize the biochemical properties of each mutant protein expressed in the baculovirus system. We have found that R82P and A172P mutants did not express well enough to enable further biochemical characterization. The 7 remaining mutants were consistently less soluble and more aggregated than WT dystrophin. We have analyzed four mutants K18N, L54R, D165V and L172H for their ability to bind F-actin and found that K18N and L54R decreased the affinity of dystrophin for F-actin by 3–4 fold. The L172H mutation affected solubility but not actin binding properties of the full-length dystrophin protein. These data suggest that disease phenotypes associated with missense dystrophin mutations are caused by either loss of solubility or a combination of insolubility and decreased F-actin affinity. We also found that mutations that cause a more severe disease phenotype (K18N and L54R) bound actin with a lower affinity and were less soluble than WT dystrophin.

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Computational modeling of the binding interaction of Jasplakinolide and Phalloidin with mammalian and parasite F-actin

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Actin,a component of the cytoskeletal system, is a polymer that is critical for maintaining the shape and motility of a cell. This is achieved by a complex dynamic regulation of rapid polymerization and depolymerization of actin. As part of its defense mechanism, certain species of fungi and marine sponges produces cyclic peptide compounds like Jasplakinolide and Phalloidin that interferes with the actin depolymerization in foreign species. In this work we use computational methods like molecular dynamics, docking and QM/MM with to elucidate the molecular details of the interaction of these compounds with mammalian and parasite actin filament. Our analysis, in addition with experimental observations from our collaborators, also helps us to propose possible mechanisms for the polymer stabilizing property of these compounds.

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Vinculin Expression Regulates Tumor Cell Invasion In 3-D Matrices

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The process of tumor metastasis formation involves cell invasion into 3-D extracellular matrices and mechanical properties of the matrices as well as focal adhesion protein complex formation are believed to regulate cell migration. We analyzed high vinculin expressing breast carcinoma cells and wildtype and vinculin-deficient mouse embryonic fibroblasts to test their ability to invade into 3-D collagen type I fiber matrices. High vinculin expressing breast carcinoma cells invaded further into collagen matrices at 2.4 mg/ml collagen concentration compared to low vinculin expressing cells, whilst at 1.2 mg/ml collagen concentration, low vinculin expressing cells invaded deeper into the collagen matrices than high vinculin expressing cells. To determine the influence of vinculin, we used wildtype and vinculin-deficient mouse embryonic fibroblasts in 3-D matrix invasion assays at these collagen concentrations. We found that the invasion depth of fibroblasts expressing vinculin was greater at high compared to low collagen concentrations, whilst vinculin-deficient fibroblasts invaded deeper into collagen gels at low compared to high collagen concentrations.
These results indicate that breast carcinoma cells and fibroblasts use at least two invasion modes which depend on collagen concentration, i.e. mesenchymal and amoeboid. In conclusion, we assume that high vinculin expressing cells follow an invasion mode at high collagen concentration through contractile force generation (mesenchymal invasion), whereas at low collagen concentration, low vinculin expressing cells follow an invasion mode at negligible contractile force generation (amoeboid invasion).

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Role of Nebulin on Actomyosin Interaction Studied *in situ* in Demembranated Skeletal Muscle Fibers from Newborn Mice

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The effects of absence of nebulin, an actin filament associated protein, on mechanical and kinetic properties of Ca^{2+}-activated, chemically skinned, psoas fibers were investigated comparing mechanical performance of fibers from 1-day-old wildtype (*wt*) mice and 1-day-old nebulin deficient ($nebulin^{-/-}$) mice. With fast mechanics (Linari et al. *Biophys J* 92:2476, 2007) on fiber bundles (sarcomere length 2.5μm, temperature 13 °C) we determined *i*) the relation between isometric force, stiffness and Ca^{2+} concentration; and *ii*) the unloaded shortening velocity and the power output at different loads at saturating Ca^{2+} (pCa, 4.50). Actin filament length in psoas fibers is not affected by the absence of nebulin, as proven by immunofluorescence imaging. Our results show a reduction in isometric force in the absence of nebulin without changes in the Ca^{2+} sensitivity of the contractile system. Stiffness measurements accompanied by analysis of the compliance of the half-sarcomere indicate that the reduction in isometric force is due to a proportional reduction in the number of myosin motors attached to actin without change in the average force of the motor. In addition, the absence of nebulin increases the unloaded shortening velocity by 63%, while decreases the maximum power output by 80%. These results indicate that the absence of nebulin induces a decrease of the rate of attachment of the myosin motors to actin and an increase of the rate of detachment of negatively strained motors under zero load, revealing a direct role for nebulin in stabilizing the actomyosin interaction. Supported by NIH and MiUR.

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Tropomyosin Specifically Regulates Type II Myosin In Yeast

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Tropomyosin (Tm) is an evolutionarily conserved α-helical coiled-coil protein that forms polymers which coil around actin filaments to regulate their integrity and function within cells. In yeast, tropomyosin stabilised actin filaments are used by molecular motors to transport cargoes or generate motile forces. Acetylation of the amino terminal methionine of mammalian Tms is required for these proteins to associate with actin and is also crucial in regulating the formation of the actomyosin complex, however it is unclear whether this post-translational modification affects myosin regulation.
The fission yeast, *Schizosaccharomyces pombe*, contains a single Tm isoform, Cdc8, which localises to the cytokinetic actomyosin ring and is absolutely required for its formation and function during cell division. Our previous work has revealed that both acetylated and unacetylated forms of Cdc8 are present within fission yeast cells, and whilst we have shown that acetylated Cdc8 is capable of regulating myosin function *in vitro,* the role of the unacetylated form remains unresolved.
S. pombe possesses five myosin's representing 3 individual myosin classes, Class I (Myo1), II (Myo2 & Myp2) and V (Myo51 and Myo52). We have examined the role that acetylated and non-acetylated Cdc8 play in regulating each class of myosin within the cell. Myo2 is found localised exclusively to the cytokinetic actomyosin ring and its function is to facilitate cell division, whilst Myo1, and Myo52 are associated with actin polymers throughout the cell cycle. In this study we show that acetylated Cdc8 specifically affects Myo2 function, but does not affect the motor activity of Myo1 or Myo52. This is not only consistent with the fact that acetylated Cdc8 is found associated predominantly with actin filaments within the cytokinetic ring, but also with the cytokinetic defects observed in cells lacking acetylated Cdc8.

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Neurospora Crassa possesses a novel ultra short tropomyosin

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Tropomyosins are alpha-helical coiled coil proteins. They interact with actin filaments; binding along the major grooves, forming a continuous filament along the actin strand. In higher eukaryotes their most well understood role is in the regulation of muscle contraction, where they regulate the myosin II - actin interaction that generates force. Their non-muscular functions are not well characterised, and in lower eukaryotes regulatory function is less clear. However, it has been clearly shown they are fundamental to maintaining the actin cyoskeleton in yeast. We have identified that *Neurospora crassa* possess two tropomyosins: a 161 residue, 4 actin spanning protein, and a 123 residue, 3

actin spanning protein. The latter isofrom is shorter than any previously identified naturally occurring tropomyosin. The only tropomyosin of similar size previously characterised was an artificial construct that was shown to possess novel acto-myosin regulatory properties. The two isoforms are produced by alternative splicing from a single gene. We have shown that the shorter isoform is expressed at much lower levels than the larger one. We have cloned the two tropomyosins and characterised their actin binding and biophysical properties. As has been found with other recombinantly expressed tropomyosins, both isoforms need the addition of an N-terminal Ala-Ser dipeptide to bind to actin. This replaces the function of the N-acetyl group present in native tropomyosins and it is hence presumed the native forms are acetylated. As flexibility has been intimately related to tropomyosin function, we determined the thermal stability of these novel tropomyosins using circular dichroism. Surprisingly this was found to be significantly higher than that of the 161 residue *S.cerevisiae* and *S.pombe* tropomyosins, and the artificial 123 residue *S.cerevisiae* construct. We are currently assessing whether this indicative measurement is reflected in the acto-myosin regulation of reconstituted thin-filaments.

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Multiple Isoforms of Fesselin (Avian Synaptopodin 2) are expressed in Smooth, Skeletal and Heart Muscle

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The synaptopodin 2 gene can be differently spliced, resulting in three mRNAs with varying 3′ ends coding for different C-termini (De Ganck et al 2008). The calculated molecular weights of these proteins are 117, 119, and 136 kDa. These isoforms require the expression of exons 1–3. However, only a single protein product has been detected in mammalian muscle lacking the product of these first exons.

We extracted four fesselin isoforms from avian smooth muscle tissue. These include the first isolated 79 and 103 kDa isoforms (Leinweber et al. 1999). The newly detected isoforms migrated on SDS gels with apparent molecular masses of 140 and 160 kDa. In contrast to our initial assumption that the 79 kDa was a proteolytic product of the 103 kDa protein we now show that they are different spliceforms. The 79 kDa isoform forms the core of synaptopodin 2. The other isoforms have different extensions at either the N- or C-terminal regions.

The different isoforms were differentially extracted by different buffers. Extractions were most complete under conditions that depolymerized both actin and intermediate filaments. Surprisingly although fesselin binds to actin and myosin none of the four different isoforms was extracted with the acto-myosin-complex. The extraction data suggest that fesselin functions in actin filament organization rather than in regulation of actin-myosin interactions. In contrast to smooth muscle tissue we detected one isoform of fesselin in skeletal and heart muscle tissue in agreement with the findings in mammalian tissue. In avian skeletal muscle we observed a 79 kDa isoform and in heart muscle a 170 kDa isoform. The reason for the differential expression of fesselin in different muscle types is unknown.

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A Mechano-kinetic Model For The Myosin-V Walking Mechanism

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The double-headed, unconventional myosin, myosin-V, transports vesicles through cells by walking toward the plus end of actin filaments in a hand-over-hand fashion. Recent single molecule experiments with high spatial and temporal resolution have elucidated a number of performance features of myosin-V that can be used to test existing models for the underlying stepping mechanism.

We present a computational model that allows us to perform detailed tests of the compatibility of existing models with known details about the mechanical and kinetic properties of myosin V. Specifically, we use a coarse-grained physical model in which the neck domains are treated as semi-flexible filaments and the lever arm rotation of the leading head is realized through state-dependent changes in the equilibrium angle between the neck and head domains. The model is well constrained by experimental data on the mechanical properties of myosin V and on the kinetic cycle, and it reproduces key performance features of myosin-V, such as the run length, the distance of the working stroke, and the stall force. It also confirms the mechano-kinetic feasibility of a proposed gating mechanism based on intramolecular strain.

Because we explicitly model the thermal motion of all motor parts, we are able to present animations of motor stepping that realistically visualize the strong influence of thermal noise on motor stepping. In addition, our model allows us to make some predictions of parameters that are yet to be measured, including details of the molecule's flexibility, and establishes experimentally accessibly performance characteristics that can be used to test these predictions.

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Regional Variations in Flexibilities Limit Continuum Rod Description of Long Coiled Coils

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Continuum rod description of coiled coils yielded a persistence length that range between 150–300 nm (Hvidt, S. et al., 1982. Biochem. 21, 4064, Adamovic, I. Et al. 2008. Biophys. J. 94, 3779). However, recent studies show that long coiled coils such as the stalk of a Rad50 complex exhibit regional variations in flexibilities (van Noort, J. et al., 2003. PNAS, 100, 7581). Apart from a length dependence due to non-bonded interactions (Lakkaraju, S. et al., 2008. BioPhys J. 94, 2404-Pos) our investigations reveal that stiffness of coiled coils is also strongly sequence dependent, hence limiting the region within which a coiled coil maybe described as a continuum rod. Bending stiffness (K_b) of a 161 Å long coiled coil with a leucine zipper periodicity calculated using normal mode analysis, forced bending dynamics and thermal fluctuation analysis (TFA) with locally built triad systems is about $1.86X10^{-27}$ Nm^2. Replacing the apolar residues at the a and d positions of the heptad repeat that are involved in the knob-into hole packing, with a hydrophilic (Glu and Lys) or polar charged residues (Ser), decreased K_b to about $0.864X10^{-27}$ Nm^2. Importantly, TFA of tropomyosin (PDB: 1C1G) mapped regions of varied flexibilities whose K_b varied between $0.33X10^{-27}$ Nm^2 (Ala 211 to Lys 221) to about $1.83X10^{-27}$ Nm^2 (Ser 36 to Leu 46), which confirms that stiffness of the structure decreases towards the C-terminus. Four of the seven actin binding sites (Brown, J.H. et al., 1986. Adv. Prot. Chem. 71, 121) lie within the regions of increased flexibility ($K_b \sim (0.75–1.36)X10^{-27}$ Nm^2), which possibly has a mechanical role during the on state of muscle contraction.

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The Striated Organelle: A Molecular Motor In Vestibular Type I Hair Cells

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The striated organelle (STO) is a structure located in the subcuticular region of hair cells, consisting of alternating thick and thin bands (Friedman, 1965; Ross and Bourne, 1983). Although present in all cochlear and vestibular hair cells, the STO is particularly well-developed in type I hair cells, where it is shaped like an inverted open cone that contacts the cell membrane along its entire circumference. It is separated from the cuticular plate by a layer of mitochondria. In other hair cells, it is a much smaller structure and appears to be free-floating. We studied its structure using electron microscopic (EM) tomography in type I hair cells. In three-dimensional reconstructions, we found that it is connected to at least some actin rootlets. It may also be associated with microtubules, mitochondria and smooth endoplasmic reticulum. Confocal immunohistochemistry places yotiao (an AKAP protein) in the same area as the STO, and the actin-binding protein, alpha-fodrin (non-erythroid spectrin), where the STO contacts the cell membrane. The contact with the rootlets suggests that the STO might regulate hair-bundle stiffness. Its association with the cell membrane suggests that the STO may help in the formation of the constricted neck characteristic of type I hair cells.

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Dystrophin and Utrophin have Distinct Effects on the Microsecond Dynamics of Actin

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This study addresses the molecular mechanisms of actin's interaction with dystrophin and utrophin, in relationship to the pathology of muscular dystrophy. Dystrophin and utrophin bind actin *in vitro* with similar affinities, but with different molecular contacts. It has been proposed that these differences alter the elasticity of actin-dystrophin and actin-utrophin linkages to the sarcolemma, affecting the cell's response to muscle stretches. To test this hypothesis, we have determined the effects of dystrophin and utrophin on the microsecond dynamics of erythrosin iodoacetamide-labeled actin using transient phosphorescence anisotropy (TPA). Binding of dystrophin or utrophin to actin resulted in significant changes in the TPA decay, revealing similarities as well as differences in the structural effects of each protein on actin. At a low level of actin saturation ($\leq$20%) both proteins induced similar changes in actin dynamics, but at higher levels of saturation, utrophin was more effective than dystrophin and induced more pronounced changes in the final anisotropy, correlation time, and initial anisotropy of actin. The simplest interpretation of these changes is that utrophin restricted the amplitude and increased the rates of motion of the probe to a substantially larger extent than dystrophin. Further analysis indicated that the actin-utrophin complex is much more torsionally flexible than the actin-dystrophin complex. We propose that these differences between dystrophin and utrophin in their effects