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Myosin tails and single α -helical domains

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Abstract

The human genome contains 39 myosin genes, divided up into 12 different classes. The structure, cellular function, and biochemical properties of many of these isoforms remains poorly characterized and there is still some controversy as to whether some myosin isoforms are monomers or dimers. Myosin isoforms 6 and 10 contain a stable single α -helical (SAH) domain, situated just after the canonical lever. The SAH domain is stiff enough to be able to lengthen the lever allowing the myosin to take a larger step. In addition, atomic force microscopy and atomistic simulations show that SAH domains unfold at relatively low forces and have a high propensity to refold. These properties are likely to be important for protein function, enabling motors to carry cargo in dense actin networks, and other proteins to remain attached to binding partners in the crowded cell.

Myosin structural diversity in humans

Myosins form a diverse group of molecular motors that are widespread [1]. In humans, the largest class is that of the 'class 2' myosins, motors that are mostly found in skeletal, cardiac and striated muscle. Myosins in class 2 include the non-muscle (NM) myosin isoforms (A, B and C) as well as skeletal, cardiac and smooth muscle myosin isoforms. They all contain a long region of coiled coil that not only dimerises the heavy chain of these myosin isoforms, but in addition, a regular repeating pattern of alternating charge along the coiled-coil tail enables the molecules to self assemble into filaments [2].

The non-muscle myosin isoforms NM2A, NM2B and NM2C form bipolar filaments [2] (Fig. 1) and NM2A and NM2B were recently shown to form co-filaments [3]. Myosin molecules pack together in an antiparallel fashion in the central region of the filament, resulting in a so-called 'bare' zone (~167nm for NM2A [2]), in which there are no myosin heads protruding from the filament. Elsewhere in the filament, the molecules pack together in a parallel fashion. Non-muscle myosin thick filaments are smaller (~300nm long [2]) than those in skeletal muscle (1600nm long), and contain fewer molecules (~30 molecules compared to 294 for skeletal muscle myosin filaments). In contrast to muscle thick filaments, the myosin heads protrude in a 'basket' like structure at their ends [2], that may facilitate a different organization of filaments with respect to those of their interacting partner, actin to that found in striated muscle.

In cells, assembly and disassembly of non-muscle myosin filaments is dynamic. For each isoform of NM2A, B and C, individual molecules form compact structures, in which the tail wraps around one motor domain, and interacts with the regulatory light chain [4]. Phosphorylation of the regulatory light chain on these molecules disrupts the head-tail interaction, activates the molecules and enables them to form filaments. The filaments can be induced to disassemble by dephosphorylation of the regulatory light chain, and a number of other regulatory mechanisms [5].

Of the remaining 11 classes of myosin in the human myosin superfamily (Fig. 1), only two further classes have been well established to contain coiled coil; myosin 5 and myosin 18. Class 5 contains 3 main isoforms; a, b and c. This myosin is distinguished by having a long lever, consisting of 6 'IQ' motifs, to which calmodulin and essential light chain (ELC) binds [6]. The long lever allows myosin 5 to take long strides as it 'walks' along actin filaments, stretching 36nm. As with non-muscle myosin 2, myosin 5 also forms a compact inactive molecule [7-10]. However, in this case, it is the binding of cargo to the C-terminal globular tail domain of myosin 5 that disrupts the head-tail interaction and allows myosin 5 to become activated. Although myosin 5 contains a coiled coil, which dimerises this myosin, it cannot form filaments. It is worth pointing out that the coiled coil between the head and the globular tail is not continuous. There is an N-terminal stretch of coiled coil just after the lever, which is about 27nm long, followed by a PEST sequence. There are then two further regions of predicted coiled coil (~12nm and 14nm long) separated by a further region that is unstructured and does not form coiled coil, leading finally to the 47kDa globular tail domain (Fig. 1). These two further regions of coiled coil have proved difficult to see in electron micrographs, and may act as an elastic element [11], enabling this myosin to traffic cargo through dense actin networks. Myosin 18a has a shorter length of coiled coil than non-muscle myosin-2 (~110nm long), and is unable to form filaments even when the regulatory light chain is phosphorylated [12]. Unusually, this myosin either has very low, or no motor activity, having lost key conserved sites for ATP hydrolysis in its nucleotide-binding site [13]. Incidentally, Myo16a, a monomeric myosin may also not have motor activity, for a similar reason [14].

Many of the remaining classes of myosin are thought to be monomeric, including the class I myosins, and those in classes 3, 9, 15 and 19 (Fig. 1). However, in three further classes of myosins; 6, 7 and 10, there is still some controversy as to whether these myosins can dimerise or not. Originally, when the sequences of these myosins were analysed using a coiled-coil prediction program such as 'Pepcoil' or 'Coils' [15], a region of sequence following the motor and lever region was predicted to be coiled coil for each of these myosin isoforms [16]. However, a close inspection of the sequence for myosin 6 showed that the predicted coiled coil of myosin 6 was rich in charge, and did not contain a hydrophobic seam [17], and moreover electron micrographs of the full-length myosin showed that it was monomeric. An examination of the predicted coiled coil domain of myosin 10 and myosin 7a again showed that they were rich in charge and lacked a hydrophobic seam [16]. The first 36 residues of the predicted coiled coil were shown to form a stable single α -

helix, that we termed 'SAH' domain [16]. We have since shown that the first 96 residues (residues 813 – 909 of bovine Myo10) are able to form a SAH domain *in vitro* [18]. However, residues 883 – 934 have been shown to form an anti-parallel coiled coil *in vitro* [19], while the isolated full length myosin 10 molecules are monomeric [20]. Moreover, it is still claimed that the proximal region of the predicted coiled coil of myosin 6 contains a region that can form coiled coil [21], even though the potential hydrophobic seam in this region is short and only likely to form a weakly interacting coiled coil [22]. Full-length myosin 7a has also been shown to be monomeric *in vitro* [23, 24].

The nature of SAH domains

SAH domains are interesting structures. Normally α -helices are only stabilized through being part of the three dimensional fold of a globular protein or as a partner in a bundle of two or more helices that form a coiled coil, where the helix is able to interact with other residues in the protein. In contrast, SAH domains are characterized by sequences that are rich in E, K and R residues and intra-molecular interactions between E and K and E and R in successive turns of the helix stabilise the helix in isolation [16]. SAH domains were first described some time ago for proteins such as ribosomal L9 and caldesmon [25, 26]. The SAH domain of L9 is quite short and the sequence is not as rich in E, K and R residues as the SAH domain of caldesmon or myosin 6 (Fig. 2A). In the longer smooth muscle longer isoform of caldesmon termed h-CaD [27], the SAH domain separates the N- and C-domains of the protein, which bind to myosin and actin respectively. An alignment of just 12 residues taken from predicted SAH domains from a variety of proteins shows how SAH domains generally are rich in E, K and R residues (Fig. 2B). SAH domains from caldesmon, L9, and myosin 6 all display significant numbers of E, K and R, often in short blocks of same-charge residues, and with (i,i+3/4) spacing between opposite charges (Fig. 2B). This pattern has been shown to enhance helicity in short artificial peptides (reviewed in [28]).

What is the function of SAH domains? Are they just a semi-flexible 'spacer' between functional domains such as the motor domain in myosin and its tail domains, or do they have other roles? We first tackled this question by determining if SAH domains can act as a lever. The canonical levers in myosin isoforms contain 'IQ' motifs to which light chains (e.g. calmodulin, regulatory or essential light chains) bind. This forms a stiff lever with a bending stiffness of about $1500\text{pN}\cdot\text{nm}^2$ [29]). When the motor domain releases phosphate following ATP hydrolysis in the nucleotide site, small conformational changes in the motor domain result in a large swing of the lever, and thus the large movement or 'stepping' of myosin along actin. The size of this step is related to the angular swing of the lever, and its length.

SAH domains as levers

Myosin 5 has a long lever containing 6 IQ motifs and this helps this motor to generate a large working stroke of $\sim 25\text{nm}$ [30]. As this motor is dimerised, it is able to walk along actin, taking steps of $\sim 36\text{nm}$ (Fig 3A). Because actin filaments have a helical repeat, and binding sites are presented to the myosin at the same azimuthal orientation only every 36nm, myosin can only walk along actin in a straight line (rather than spiralling around the filament) if it binds to actin ever 36nm, reviewed in [31]. Moreover, myosin 5 can walk 'processively' along actin, taking multiple steps without detaching. This behaviour arises from its unusual ATPase cycle, where release of ADP, rather than P_i , is the rate-limiting step (slowest step) in the ATPase cycle. The result of this is that myosin 5 spends most of its ATPase cycle bound to actin (in contrast to skeletal muscle myosin, in which P_i release is rate limiting, and the myosin spends most of its ATPase cycle detached from actin). However, as the walking stride (36nm) is larger than the working stroke ($\sim 25\text{nm}$), when two heads are bound to the actin filament at the same time, the interaction between two heads (Fig. 3A, steps 2-3) prevents the leading head from completing its powerstroke (i.e. the movement of its lever from pre- to post powerstroke position). This is associated with a very slow rate of ADP release from the leading head compared to the rear head or a single head [32, 33], a process known as 'gating' of ADP release. However, when the rear head binds ATP and detaches (Fig. 3A: steps 4-5), the leading head can then execute its powerstroke, and this 'swings' the detached rear head forwards to find the next binding site on actin, and to become the new leading head. This process additionally biases continuous forward movement along the actin filament.

The long lever length is required for this myosin to be able to take 36nm steps as it walks along actin, and shortening the lever to just 2 IQ domains (from 6) reduces the working stroke and the molecule cannot take many steps along actin [34]. We tested if the SAH domain can contribute to the lever function of this myosin, by replacing 4 of the 6 IQ motifs with a SAH domain, (i.e. a 2IQ motor, with a SAH domain equivalent in length to 4 IQ motifs, Fig. 3B) in a myosin 5 molecule and

asking if the motor could still walk along actin in a similar manner to the wild type molecule. If the SAH domain could not function as a lever, we would expect the behaviour of the chimeric molecule to be similar to that of myosin 5 with only 2IQ motifs. However we found that it was able to walk along actin in a similar way to wild type 6IQ myosin 5 [23]. Thus the SAH domain can act as a lever. Interestingly however, the chimeric myosin 5 motor was no longer 'gated', and ADP was released at the same rate from both heads. What this means is that there is now a 50% chance that the leading head can bind ATP and detach before the rear head, and if it does so, it can then re-attach to the same binding site, and the myosin will not move forwards (Fig. 3A, steps 2 → 6 → 1). Therefore, inserting a SAH domain into a genuinely dimeric motor has the disadvantage that the motor will move along actin more inefficiently, as about 50% of the time, the lead head will detach before the rear head, and re-attach at the same binding site. It is only by biasing detachment towards the rear head that the motor is able to continue to walk forwards, as found in the wild type myosin 5. Thus, while the SAH domain can contribute to lever function, its reduced stiffness compared to a canonical lever (~10 times less stiff [23]) means that it is unable to produce gated ADP release between heads in a dimeric motor. The SAH domain from myosin 10 has since been shown to act as a lever in an artificially dimerised myosin 10 motor, 3IQ & SAH containing construct [35].

SAH domains as constant-force springs

As already mentioned, SAH domains are not just found in myosins. Based on sequence analysis, they are likely to be found in a range of proteins [28, 36, 37]. Clearly they could act as 'semi-flexible' linkers in these proteins, but do they have an additional role? We questioned whether the SAH domain might also be able to unfold under low forces acting as a 'constant force' spring in proteins. Proteins in cells need to work in a crowded environment, as nicely illustrated by Goodsell and others [38, 39]. A typical mammalian cell has a volume of 4pL and more than 20% of this volume consists of proteins, with 10,000 to 20,000 different proteins in a cell. Actin is present at about 4mg/ml (25 μ M) in cells, for example. Other proteins, including many myosin isoforms are present at much lower concentrations (~1000 molecules per cell – ~0.4nM [40]). Moreover, we know that forces affect the functions of motors. Pulling backwards on motors reduces their forward velocity and a high enough force will prevent forward movement (the 'stall force'; where forward and backward stepping rates are equal), at which the motor may even detach. The stall forces for myosin 5 and 10 are quite low; 1-3 pN [30, 41], and is also low for non-muscle myosin 2 (~2pN for NM2B [42]). Therefore, a reversibly extensible linker between the motor domain and the tail might also be useful for myosins that experience forces above or close to stall forces in cells.

We used AFM and atomistic simulation to understand the behaviour of SAH domains when they experience extension forces. We found that these domains unfold at low forces (less than 30pN) when pulled at 1000 nm s⁻¹ *vitro*, and our data suggested that these domains unfold non-cooperatively such that as soon as force is applied and the protein starts to unfold, the force generated by the protein itself stays roughly constant [18]. This is in contrast to a typical 'Hookean spring' where the force would increase linearly as the protein lengthens [43]. *In vivo*, we imagine that as the motor is attempting to pull its cargo forward, if the cargo becomes trapped, and the force experienced by the motor then increases (Fig. 3C), the SAH domain can start to unfold. This would allow the force to remain constant and low, allowing the motor to remain attached to its track until the cargo is released, and the motor can continue to carry it forward. Simulation shows that the SAH refolds completely and fast, i.e. without encountering a free energy barrier, after the force is released [18].

SAH domains could help myosins and other proteins in particular situations

It is interesting that three different types of myosins in the human genome contain (6, 10) or are likely to contain (7a) a SAH domain. Myosins 7a and 10 are related, in that they have similar tail domains. They are both known to function in regions of the cell that have high actin filament density such as the filopodia for myosin 10 [44, 45] and stereocilia for myosin 7a [46]. Moreover, myosin 6 is also known to associate with regions of high actin density such as the pericuticular necklace of inner hair cells (a junctional actin belt [47]). The SAH domain may therefore facilitate the continued association of the motor with its track in regions of high actin density where otherwise forces applied to the tail might detach it, or affect its ability to move along actin filaments. Could SAH domains perform a similar function in other proteins? We, and others have identified potential SAH domains in many different proteins from bacteria to humans [28, 36, 37]. The SAH domain is nearly always sandwiched between two or more functional domains. It is possible that

the SAH domain again prevents the detachment of these functional domains from their binding partners when force is applied that drives the binding partners apart, while also providing a low restoring force to allow re-establishment of the status quo once that extraneous force has dissipated.

In conclusion, myosins are diverse, and adapted for many different cellular functions. Myosins that contain SAH domains may be particularly well adapted to work in cellular regions with dense actin bundles or network. By sacrificial unfolding of the SAH domain under low forces, the detachment of the motor from its track may be prevented when it experiences external force; similar behaviour may also occur in other proteins containing SAH domains.

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Figure Legends

Figure. 1. Human myosin isoform diversity. There are 12 classes of myosins in the human genome. The structure and function of these myosins is highly varied, as illustrated here.

Figure 2: SAH domains A) The SAH domain from L9, one of the first stable α -helices to be characterized (see text); part of the SAH domain from human caldesmon, which has a repeating sequence, and the SAH domain from myosin 6. Potential $i, i+4$ ionic interactions are shown as brackets between residues above the sequence, and potential $i, i+3$ interactions as brackets below. B) An alignment of 12 residues taken from part of a predicted SAH from a variety of proteins. The Uniprot reference is given for each one. The alignment shows the typical feature of blocks of charged E,K and R residues.

Figure 3. The SAH domain as part of the lever in myosins. A. The ATPase cycle for myosin 5, with the prominent 'normal' cycle as steps 1-5. Less common is the step from 2 to 6, where the front head loses ADP before the rear head, can then bind ATP and detach, then reattach in the same position (a 'futile' cycle). Gating biases the cycle towards steps 1-5 to promote forward motion. If 4 of the 6 IQ motifs are replaced by a SAH domain (illustrated in B), then steps 2-6 become more frequent, with a 50:50 chance of a molecule at step 2 progressing to step 3 or to step 6. This is because the SAH domain is not stiff enough to produce gating of ADP release by the two motor domains, which normally biases release of ADP from the rear head. C) In a monomeric myosin, as the myosin completes its powerstroke, an external force pulling on the tail that might prevent this occurring, results in a partial unwinding of the SAH domain. Subsequently the external force is removed, the SAH domain refolds, and the tail plus any cargo is able to move forward.

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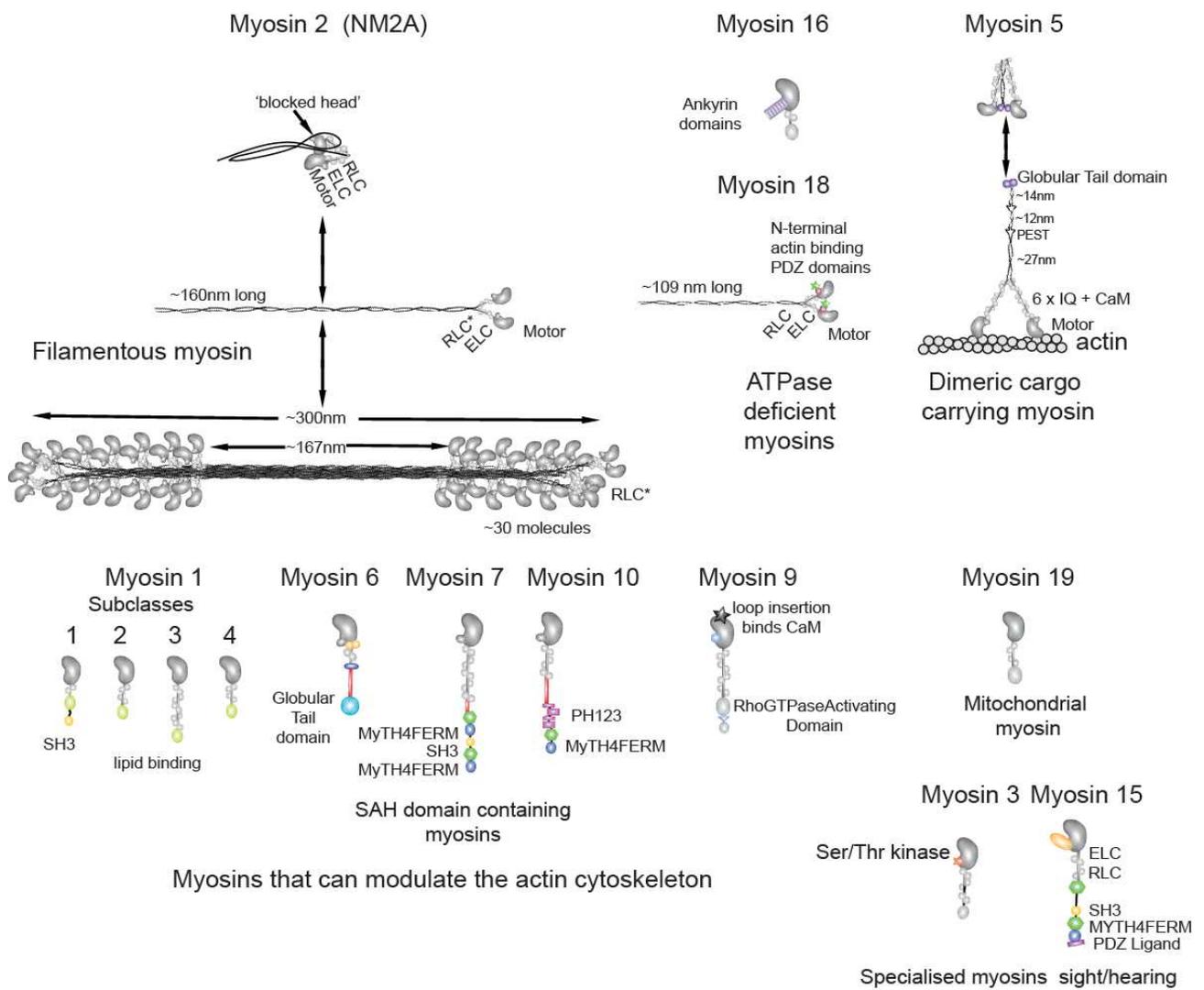
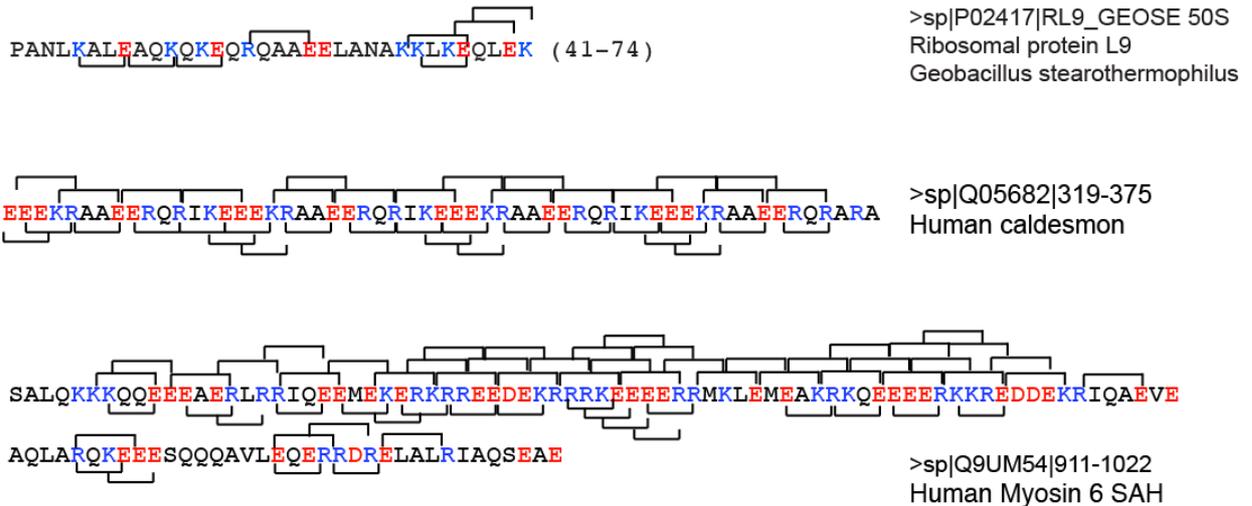


Fig. 1. Human myosin isoform diversity. There are 12 classes of myosins in the human genome. The structure and function of these myosins is highly varied, as diagrammed here.

A



$i, i+4$ interactions are shown above the sequence 
 $i, i+3$ interactions are shown below the sequence 

B

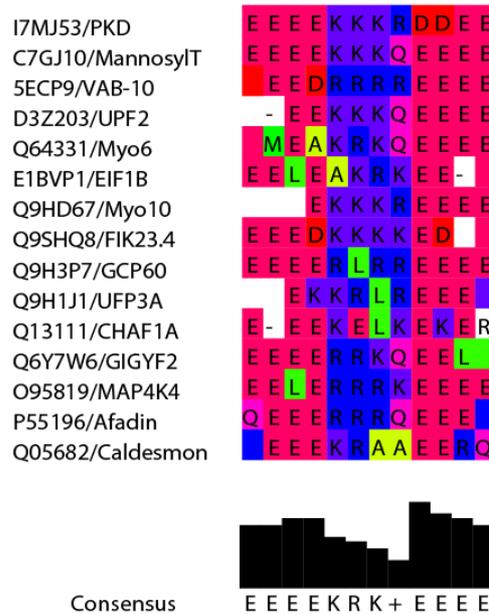


Figure 2: SAH domains A) The SAH domain from L9, one of the first stable α -helices to be characterized (see text); part of the SAH domain from human caldesmon, which has a repeating sequence, and the SAH domain from myosin 6. Potential $i, i+4$ ionic interactions are shown as brackets between residues above the sequence, and potential $i, i+3$ interactions as brackets below. B) An alignment of 12 residues taken from part of a predicted SAH from a variety of proteins. The Uniprot reference is given for each one. The alignment shows the typical feature of blocks of charged E, K and R residues.

