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Calcium and cargoes as regulators of myosin 5a activity

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Abstract

Myosin 5a is a two-headed actin-dependent motor that transports various cargoes in cells. Its enzymology and mechanochemistry have been extensively studied *in vitro*. It is a processive motor that takes multiple 36 nm steps on actin. The enzymatic activity of myosin 5 is regulated by an intramolecular folding mechanism whereby its lever arms fold back against the coiled-coil tail such that the motor domains directly bind the globular tail domains. We show that the structure seen in individual folded molecules is consistent with electron density map of two-dimensional crystals of the molecule. In this compact state, the actin-activated MgATPase activity of the molecule is markedly inhibited and the molecule cannot move processively on surface bound actin filaments. The actin-activated MgATPase activity of myosin 5a is activated by increasing the calcium concentration or by binding of a cargo-receptor molecule, melanophilin, *in vitro*. However, calcium binding to the calmodulin light chains results in dissociation of some of the calmodulin which disrupts the ability of myosin 5a to move on actin filaments *in vitro*. Thus we propose that the physiologically relevant activation pathway *in vivo* involves binding of cargo-receptor proteins.

Keywords: Myosin; Calmodulin; Regulation; Calcium

Myosins are actin-dependent motor proteins that perform a variety of tasks inside cells including contraction of muscle, division of cells during cytokinesis, movement of intracellular organelles and molecules, and maintenance of cell adhesion. There are now more than 25 classes of myosins known based on sequence analysis of their motor domains. Humans have approximately 39 myosins from 12 of these classes. Professor Ebashi had a fascination with calcium ions and their role in regulating muscle contraction and other cellular events. It is therefore relevant that many of the unconventional myosins have calmodulin as a light chain, giving rise to the possibility of calcium being a regulatory switch to control their activities. The mechanism for the regulation of the activity of many of types of myosins has been studied. In several cases, calcium ions are involved either directly or indirectly. For example, scallop muscle myosin (belonging to the class 2 myosins) is regulated by the binding of calcium to one of the light chain subunits that bind to the neck region [1]. In the absence of calcium the MgATPase of the myosin is not activated significantly by actin. Upon binding of calcium the MgATPase activity of the myosin is greatly activated by actin. The affinity for calcium is in the 0.1–1 μM range and the on-off switch defined by the binding and dissociation of calcium is rapid, as would be expected of the main regulatory switch for a fast contracting muscle. In other cases, the activity is regulated by phosphorylation of either the myosin heavy chain or of a light chain subunit. An example of the first case is certain myosin 1 molecules from lower eukaryotes such as *Dictyostelium discoideum* or *Acanthamoeba castellanii* [2]. Here, a serine or a threonine residue in a surface loop thought to directly interact with

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actin is phosphorylated, resulting in a dramatic increase in the actin-activated MgATPase. Another example of a phosphorylation-dependent regulation is vertebrate smooth muscle and nonmuscle myosins 2. In both these cases, the regulatory light chain is phosphorylated by kinases, resulting in a change from an inactive to an active form of the myosin [3]. In marked contrast to these regulated myosins, vertebrate skeletal muscle myosin 2 is constitutively active and its interaction with actin is regulated by the troponin–tropomyosin complex on the thin filament, as was first shown by Ebashi's group [4].

In this article, we will focus on the regulation of myosin 5a, a molecular motor that moves cargo along actin filaments in cells. While calcium has an effect on the actin-activated MgATPase and the motility, we argue that it is probably not the physiological mechanism of regulation. Instead, a quite different mechanism is used that directly senses the binding and release of the cargo that the molecule transports. There are three myosin 5 genes in mammals, termed 5a, 5b, and 5c [5]. Of these, myosin 5a is best characterized in terms of its enzymatic and mechanical properties as well as its intracellular roles [6,7]. Myosin 5a transports melanosomes in melanocytes and also plays a role in neuronal transport [8]. It is this species we concentrate on in this article. Myosin 5a is a two-headed motor which moves processively on actin [6]. Each of the two amino-terminal motor domains are followed by a long neck region containing six IQ motifs which bind calmodulin or, in some cases, calmodulin-like light chains in the absence of calcium ions. Carboxy-terminal to the neck regions, the two heavy chains dimerize via a coiled-coil which is followed by paired globular tail domains.

Calcium regulation of myosin 5a is paradoxical

The early characterizations of myosin 5a purified from chicken brain showed that its actin-activated MgATPase activity was greatly activated by micromolar levels of calcium ions. Paradoxically, calcium inhibited the ability of the myosin to move actin filaments in an *in vitro* motility assay in which the myosin was bound to a surface [9]. These findings were reproduced and extended by more recent studies [9–12]. Calcium also has a large effect on myosin conformation as determined by analytical ultracentrifugation [10–12]. In the presence of calcium or at high ionic strength, myosin 5a sediments at 11S. In contrast, when calcium ions are depleted, myosin 5a sediments at 14S. These conformational changes have also been demonstrated by electron microscopy. The 14S myosin has a compact, folded conformation very different from the open conformation seen at high ionic strength or in the presence of calcium (Fig. 1A and B).

Myosin 5 turns itself off

A carboxy-terminally truncated myosin 5 equivalent to heavy meromyosin (HMM) does not show the same calcium-dependent changes in S value as observed with full length myosin 5. Moreover, the actin-activated MgATPase activity of myosin 5a HMM is high regardless of the calcium level. These observations suggest that a head–tail interaction is necessary for adoption of the compact, folded conformation and that the folded conformation represents the "off" state of myosin 5a [10,11]. Addition of an expressed GST-globular tail domain (GTD) fusion protein suppresses the enzymatic activity of myosin 5a HMM with a K_d of around 1 μM. In addition, electron micrographic images show that in the absence of calcium myosin 5a HMM and the GTD form a folded complex very similar to that of the intact molecule [13,14] (Fig. 1C and D). GST dimerizes and thus this fusion protein contains two GTD domains per molecule which presents itself in a similar manner to the native GTD domain present at the end of the dimeric coiled-coil tail of myosin 5a.

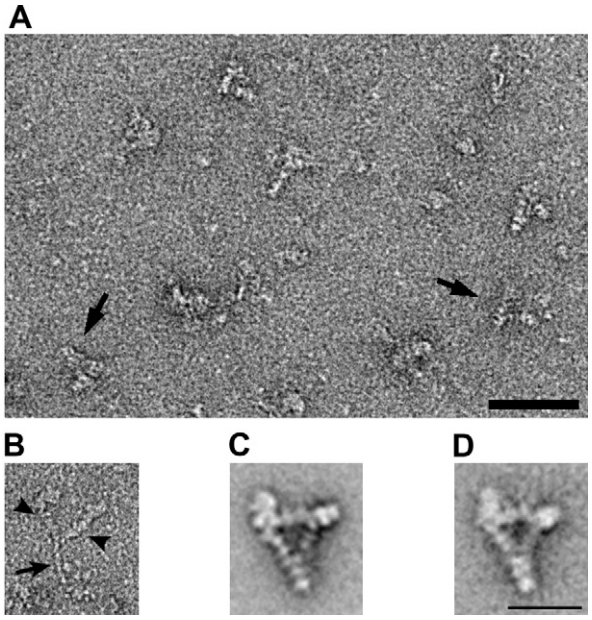


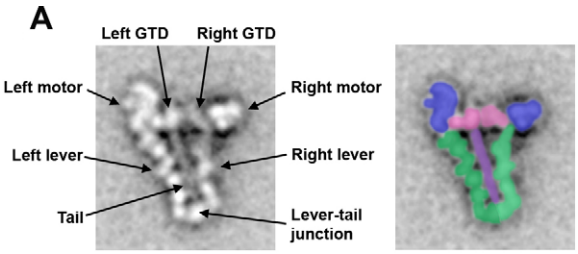
Fig. 1. Negative stain electron microscopy of myosin 5a. (A) A field of myosin 5a under switched off conditions, showing triangular, folded molecules (arrows). (B) A molecule opened up by high salt concentration, showing two heads (arrowheads) and coiled-coil tail (arrow). (C) An averaged image of folded molecules. (D) An averaged image of myosin 5a HMM molecules complexed with dimers of a GST-globular tail domain fusion protein; note the similarity to (C). Scale bar for (A) and (B), 50 nm; for (C) and (D), 20 nm.

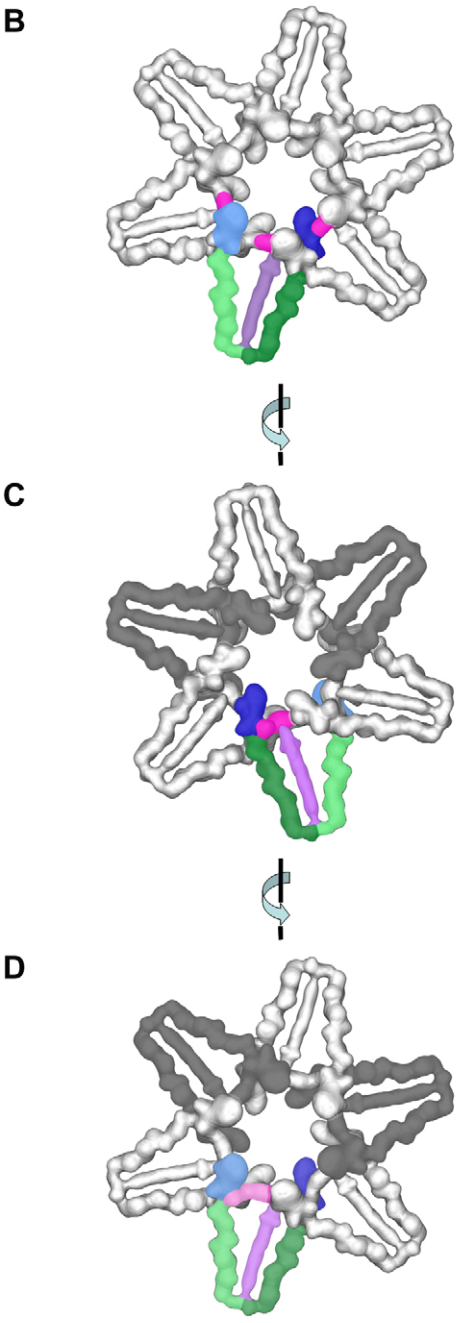
Structure of switched-off myosin 5

Detailed images of the folded, regulated molecules have recently been published by two groups. One study used single particle averaging of individual negatively-stained myosin 5a molecules [13] (Fig. 2A). The other analyzed EM images of two-dimensional crystals of myosin 5a formed on positively charged lipid monolayers. The crystal packing has groups of six folded myosin 5a molecules forming a rosette-like structure (Fig. 2B) packed in a hexagonal lat-

tice [15]. Cryo-EM yielded a three-dimensional density map of the rosette that was interpreted as showing that the heads of one myosin 5a made contact with the GTD domains of the adjacent myosin 5a molecules on either side. It was supposed that this 'domain swapping' between molecules occurred during assembly of the crystal. Both studies show that the two heads interact with the GTD and the negatively-stained images of single myosin 5a molecules show clearly that this is an intramolecular interaction [13]. However, the studies differed on the location of the contact between the head and the tail. The crystal study suggested that the GTD interacts with the loop 1 region of the head, but the single molecule study suggested that the tail docking region of the head was a surface helix–loop–helix fold (amino acids 117–137) that has two negatively charged amino acids that are highly conserved within the myosin 5 subfamily, but not found in other myosin isoforms [13] (Fig. 3). In both cases the predicted interaction site is relatively close to the nucleotide binding pocket, but is not in close proximity to the actin binding site. By comparison with atomic structures the myosin heads appear to be in a "post rigor" conformation which would be predicted to bind weakly to actin.

We show here a new comparison between the two reported structures that suggests the rosette can be reinterpreted as built from six folded molecules without domain swapping, and with the GTD interacting with the same part of the motor domain as seen in the single molecules.





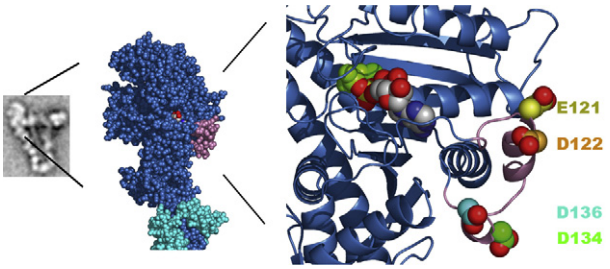


Fig. 3. Detail of the GTD binding site on the myosin 5a motor domain. Left panel: an averaged image of folded molecules; the GTD is very clear on the left motor domain. Centre panel: atomic structure of myosin 5a head with ADP and BeF_3 bound in the active site (red) (1w7j.pdb) [35]. The first light chain is colored cyan, and the GTD binding region (residues 117–137) is mauve. Actin binds to the top left region. Right panel: ribbon depiction of part of the motor domain. The conserved acidic residues and the nucleotide are shown in spacefill. Loop 1 can be seen above E121.

Fig. 2. Structure of regulated myosin 5a. Motor domains are shaded blue, levers green, coiled-coil tail mauve and globular tail domain (GTD) pink. (A) Averaged image of separate folded molecules, labeled at left and colored at right [13]. (B) 3-D structure from cryo-EM of rosette of six molecules, partly colored according to interpretation of Liu et al. [15]. The motors were proposed to interact with GTDs of adjacent molecules by 'domain swapping' between molecules. (C) Rosette structure flipped over around a vertical axis, and reinterpreted to match (A). Motor domains are proposed to interact with their own GTDs, and part of each molecule lies under the next molecule anti-clockwise round the rosette. As a result the right GTD and most of the right motor is hidden under the left motor and GTD of the adjacent molecule. Two molecules are shaded darker grey to emphasize the boundaries between the six molecules. (D) Reinterpreted rosette structure flipped over again for comparison with the original interpretation (B). Note that parts of the GTD identified in this view were assigned to two motor domains in the original interpretation.

This reinterpretation arises most naturally when the rosette is flipped over from the view published, as the tail is then adjacent to the left lever as in the single molecules (Fig. 2C). Features closely matching the left motor and associated left GTD of the single molecule are clear. The right motor lies under the adjacent molecule in this view, but it is roughly end on, just as in the single molecule. Flipping the rosette back to the original view (Fig. 2D) shows how the reinterpretation fills the densities with features of single folded molecules. An attractive feature of the reinterpretation is that crystallization is then seen to have occurred without disruption of the native regulatory contacts between GTD and motor.

A new puzzle concerns the structure of the coiled-coil tail. Analysis of the amino acid sequence predicts there would be about 57 nm of coiled coil, broken into three segments by non-helical sequences. Early shadowed images were broadly consistent with this although the length was variable [9]. In the folded molecule only the first segment, about one third of the total, was seen, either in single molecules or in the 2-D crystals. This suggests the other segments are either unstable or very flexible. It has therefore come as a surprise that the off state is very sensitive to the length of this invisible part [14].

Does switched-off myosin 5a recycle via the actin treadmill?

One group [15,16] proposed a method for recycling the myosin back towards the middle of the cell when it was not bound to the cargo. They speculated that the folded, inactive myosin 5a not bound to cargo might bind to actin filaments undergoing retrograde flow. Evidence for this was that in the presence of ADP and phosphate, the folded conformation of myosin 5a bound to actin. Other evidence argues against this model. In the presence of ATP (as in the living cell) the folded myosin 5a (probably as a myosin.ADP.Pi complex) binds only weakly to actin, as seen by electron microscopy and TIRF microscopy [13], and from enzymatic studies [17–19]. Thus, riding an actin treadmill would not return the inactive myosins to the cell center. Moreover, cell biological studies of mutant melanocyte cells lacking the protein that connects myosin 5a to its cargo, show that myosin 5a is diffusely localized in cells and is not preferentially associated with actin filaments [20].

Why does calcium inhibit myosin 5 motility?

The basis for the calcium-dependent inhibition of the mechanical ability of myosin 5a has been explored in more detail [21,22]. The impairment is likely a consequence of a calcium-induced dissociation of one or more calmodulins from the neck region. ''Naked'' IQ motifs are known to compromise the movement of myosin, probably by rendering the lever arm floppy [23]. Calcium-calmodulin binds with a significantly lower affinity to some IQ motifs, particularly IQ2 of mouse myosin 5a [24,25]. Single molecule motility studies show that calcium-induced calmodulin dissociation causes a dramatic shortening of the run length, which can be partially rescued by inclusion of 12 μM calmodulin in the assay [21].

Cargo binding as a regulatory switch

The best studied physiological function of mammalian myosin 5a is its role in transporting pigment granules, termed melanosomes, in melanocytes [26]. It was first characterized as the product of the *dilute* gene in mouse which when mutated gave rise to pigmentation defects [27]. Melanosomes are made in the cell body and then transported out to the tips of dendritic processes in a cooperative process requiring both the microtubule based motors, dynein, and kinesin, as well as myosin 5a. All three motors are bound to the same melanosomes. The long range motions are carried out on microtubules which are present along the length of the dendrites. The tips of the dendrites are rich in actin filaments and it is thought that upon reaching this area, myosin 5a engages the actin and effectively ''docks'' the pigment granule. This process was termed the ''cooperative capture model'' [26]. Melanocytes cultured from *dilute* mice have robust bidirectional transport of pigment granules along the microtubules, but fail to capture the pigment granules in the dendritic tips due to lack of myosin 5a.

Studies on two other mouse pigmentation mutants reveal the nature of the cargo-receptor complex. A protein, termed melanophilin, which is the product of the mouse *leaden* gene directly binds to the tail region of myosin 5a. Melanophilin, in turn binds in a GTP-dependent manner to Rab27a, a membrane bound protein tightly associated with melanosomes. Rab27a is the product of the mouse *ashen* gene [20,28–30]. This tripartite cargo-binding complex was recently reconstituted in the single molecule motility assay [31]. A GFP-tagged Rab27a fusion protein was mixed with unlabeled melanophilin and myosin 5a. Fluorescent spots could be observed moving along actin filaments at the characteristic rate for myosin 5a. Movement of the spots was dependent upon having both myosin 5a and melanophilin present as well as ATP [31].

Solution of the calcium paradox

These insights bring us back to the question of why does myosin 5a move actin filaments in the absence of calcium when it is bound to a coverslip in the sliding actin *in vitro* motility assay? The most reasonable answer to this question is that upon binding to the coverslip surface in the absence of calcium, some or all of the myosins become unfolded and thus active. In other words the coverslip surface mimics binding to cargo. Another widely used motility assay follows the movement of single fluorescently-labeled molecules of myosin 5a along actin filaments which are bound to the surface. This is the assay that is most commonly used to measure the degree of processivity of myosin

5a since run lengths can be measured. Several studies have used this assay to study the movement of full length myosin 5a. These studies are typically conducted in the absence of calcium since calcium would inhibit the movement of the myosin as previously discussed and this raises the issue of how a myosin which is supposed to be in the folded, off state moves along actin. We tested this by comparing the frequency of movement of Cy3-labeled full length myosin 5a with that of a GFP-labeled HMM fragment of myosin 5a in the absence of calcium. We found that the frequency of movement of full length myosin was 40 times less than that of the HMM demonstrating that most of the molecules of full length myosin 5a are inactive in solution in the absence of calcium [13]. The movement events that are observed could be from a small percentage (2–3%) of the myosin that is damaged and cannot assume the folded, off state. Alternatively, since it is likely that the folded and extended forms of myosin 5a are in equilibrium, the rare movement events that occur with full length myosin could be due to myosin molecules which happen to be extended at the instant that they collide with an actin filament.

Thus, calcium is probably not the physiologically relevant regulator of myosin 5a. Although calcium does activate the actin-activated MgATPase activity of myosin 5a, it would result in poor mechanical performance unless free calmodulin concentrations were very high. Instead, it is more likely that binding of a cargo receptor, like melanophilin, to the globular tail domain of myosin 5a activates its enzymatic and mechanical activity by sterically or allosterically disrupting the interaction between the motor domain and the tail [31,32]. According to this model, only occupied motors would be active in cells. Calcium could be playing a role by allowing the myosins to remain engaged in a nonproductive manner with actin filaments [12]. Virtually all of the unconventional myosins have calmodulin or calmodulin-like proteins as light chains. In some cases calcium binding does not result in calmodulin dissociation and has been shown to play at least a modulatory role in controlling the enzymatic activity [33,34].

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