Calcium gets myosin VI ready for work

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The myosins are a large family of actin-binding motor proteins that convert stored chemical energy into work, with important functions in intracellular transport, force generation and mechanosensation (1). Despite many advances in understanding the mechanical and kinetic properties of purified myosins *in vitro*, the signals that regulate the functions of these molecular motors in cells are not well characterised. In this issue of PNAS, Batters et al. (2) identify a key regulatory function for the universal intracellular signal calcium (Ca^{2+}) in the control of myosin VI activation. Myosin VI is the only myosin that moves toward the minus end of actin filaments and contains many unusual structural features (3). Batters et al. show that for this myosin, Ca^{2+} links motor activation to cargo binding in a step-wise process by controlling the orientation and interactions of its binding protein Calmodulin (CaM). Whilst Ca^{2+} has been shown to exert structural effects on other myosins, this paper unravels another distinct and unusual mechanism employed by this most unconventional myosin.

All myosin motors contain essentially three domains; a highly conserved N-terminal actin binding head domain, a lever-arm and a tail domain, which may contain coiled-coils that mediate dimerization and/or specialized cargo-binding domains (CBD). The N-terminal head domain is an actin-activated ATPase that undergoes conformational changes coupled to the hydrolysis of ATP (4). The lever arm, typically containing one or several IQ motifs, amplifies these relatively small (Angstrom) movements within the head domain into nanometer displacements of the tail. Association of the Ca^{2+}-binding protein CaM with the IQ motifs confers structural stability to the lever arm, enabling mechanical coupling between the head and tail domains. Variations in ATPase kinetics and lever arm structure and length across the different myosins translate into different molecular functions such as transport or tethering. In cells, the localisation of each myosin is determined by their highly divergent cargo-binding tails. Myosin VI has been shown to associate with a large number of different cargo-adaptor proteins through binding to specific sites on its CBD, giving rise to the concept that a single motor may have a multitude of cargoes and functions (5). Despite much progress, there are still many unanswered questions about the cellular regulation of myosin motors. For example, motor activation may result from an unfolding transition (6), cargo-mediated dimerization (7) or recruitment of CaM (8). However,

the spatiotemporal regulation of myosins and the co-ordination of motor activation and cargo attachment are poorly understood.

To investigate how Ca^{2+} might regulate the lever arm of myosin VI, Batters et al. (2) employed single particle electron microscopy to visualise thousands of individual molecules. They found that the orientation of the single CaM on the lever arm of myosin VI was sensitive to Ca^{2+}. In the absence of Ca^{2+} the electron density was consistent with the arrangement seen in previous structures (9). However, addition of Ca^{2+} led to a rotation of CaM by 30°. What was the cause of this rotation? By using a library of peptides representing sections of the myosin VI lever arm and examining their binding affinity in the presence and absence of Ca^{2+}, they found that a single Ca^{2+}/CaM can simultaneously bind to both the IQ domain and to an additional site separated by an alpha helix, forming a destabilising 'bridge'. In contrast, apo-CaM (i.e. Ca^{2+} free) only binds to the IQ domain. Thus the lever arm CaM binds to myosin VI in two different modes, depending on Ca^{2+} concentration.

A striking feature of myosin VI is that it has a step size almost identical to myosin V (36 nm), but a much shorter apparent lever arm, containing only a single IQ motif in contrast to the six IQs of myosin V. Structural work has determined that the proximal tail (amino acids 835-907), located at the C-terminal end of the myosin VI IQ domain, contains a three helix bundle (3HB) (9, 10) and unfolding of this bundle creates a lever arm extension that accounts for the observed step size of myosin VI (9). To explain how the 3HB can be rigidified to serve as a lever arm, a recent study proposed that an additional CaM binds to the loop between helix 1 and helix 2 of the 3HB (8). The second part of the bipartite binding site for Ca^{2+}/CaM identified by Batters et al. in the primed conformation is in just this very loop. However, Batters et al did not observe binding of an additional CaM, rather, the lever arm CaM re-orients itself to bind to both sites in the primed state. At present, the structural adaptations that would allow an additional CaM to occupy the hinge binding site in full-length dimerized myosin VI (8) are unclear. Of note, in the model of Batters et al., the rotation and bridging of CaM occurs prior to cargo binding, whereas the proposed stabilisation of the 3HB by binding of an additional CaM (8) occurs after cargo binding. This latter process might be coupled to the lowering of free Ca^{2+} and the transition to the motile state described in the model (2).

In the model of Batters et al. in low Ca^{2+}, apo-CaM simultaneously binds to both the IQ domain and part of the CBD in the tail, keeping myosin VI in a folded, inactive conformation (2). This is consistent with a previous report of a closed conformation of this myosin (10). Interestingly, the region of the CBD that interacts with the apo-CaM contains the adaptor protein-binding RRL motif and the phospholipid binding site (Figure 1), thus blocking the major cargo binding domains. In the presence of Ca^{2+}, myosin VI adopts an open 'primed' conformation, releasing the tail from the IQ-bound

Ca^{2+}/CaM with the CBD becoming accessible. This fits with a previous report that myosin VI binds to phospholipids only in the presence of Ca^{2+} (11). Of note, there is no motility (as measured by filament gliding assays) in the presence of Ca^{2+}. Further analysis of EM images showed that this was due to a much higher flexibility in the lever arm at high Ca^{2+}, meaning mechanical work by the head could not be transduced to the tail, despite the fact that the Ca^{2+}/CaM did not detach from the lever arm.

Folding-unfolding transitions have been observed in several other myosins (Figure 1) including myosin V (12), myosin VII (13) and myosin X (14) (those in myosin V and myosin VII are also Ca^{2+}-dependent), and may be important regulatory steps in cells to prevent their translocation on actin in the absence of useful cargo transport (this mode of regulation has also been observed in the microtubule motor kinesin (15)). The conformation of non-muscle myosin II has also been shown to be regulated by Ca^{2+}, which activates the kinase MLCK to phosphorylate the light chains allowing the myosin II tail to unfold and assemble into actin-binding filaments (16). The priming of myosin VI by Ca^{2+} however is unique among myosins, and adds to the complex picture of motor regulation. Importantly, Batters et al. provide a testable model that makes predictions about myosin VI function. However, many questions remain unresolved:

(1) The region of the CBD (1060-1125) that interacts with apo-CaM contains the RRL motif, the binding site for adaptor proteins such as Optineurin and GIPC (17), and the phospholipid-binding motif. Future experiments should examine which residues in the CBD interact with apo-CaM and identify mutations that affect the activity of myosin VI *in vivo*. What role do cargo-adaptor proteins play in the transition from the non-motile to the motile form? Excess CaM is required to reconstitute full myosin VI motility in vitro, and so binding of a third CaM may be required to stabilise the lever arm extension once Ca^{2+} is reduced (8).

(2) In contrast to myosin V, regulation of myosin VI activity does not involve binding of the tail to the motor domain but instead to the lever arm. Therefore, can the head domain of inactive myosin VI still bind to actin? This might allow the switched-off myosin VI to be parked on actin filaments with translocation initiated by cargo binding. Alternatively, in which of the different steps in the activation cycle can the motor bind to actin filaments; in the back-folded, primed, cargo-bound non-motile or only in the cargo-bound motile conformation?

(3) How does myosin VI refold at low Ca^{2+}? Is cargo release the trigger that allows the tail to back-fold onto CaM?

(4) In which physiological situations can Ca^{2+} regulate myosin VI function? In neurosecretory cells, recruitment of myosin VI to secretory granules depends on Ca^{2+} (18) and there are also emerging roles

for Ca^{2+} in spatially restricted systems where myosin VI has been shown to function (19), for example in autophagy (20). Ca^{2+} signals may also be important in regulating unconventional myosin function in cardiac tissue and in adaptation of the stereocilia in the inner ear. Improved tools for the detection and manipulation of transient localised Ca^{2+} signalling in mammalian cells may also shed further light on the co-ordination of myosin motor proteins.

In summary, the contribution of Batters et al. (2) is provocative and raises many questions, which should stimulate further studies probing Ca^{2+} regulation of myosin VI function in a wide spectrum of cellular processes.

Figure 1. Cargo-interaction motifs on the myosin VI tail and folding-unfolding transitions for several myosin family members; myosins II, V, VI, VII and X. Myosin VI cargoes are listed below their respective binding motifs (RRL or WWY). The large insert (LI) present in the construct used by Batters et al. is shown in purple. Key to the folding transition cartoon; red regions indicate motor domains, blue are IQ motifs, green are cargo-binding domains and orange circles show phosphorylation sites. The curved surfaces indicate cargoes.

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