Regulated Conformation of Myosin V*

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We have found that myosin V, an important actin-based vesicle transporter, has a folded conformation that is coupled to inhibition of its enzymatic activity in the absence of cargo and Ca2+. In the absence of Ca2+ where the actin-activated MgATPase activity is low, purified brain myosin V sediments in the analytical ultracentrifuge at 14 S as opposed to 11 S in the presence of Ca2+ where the activity is high. At high ionic strength sediments at 10 S independent of Ca2+, and its regulation is poor. These data are consistent with myosin V having a compact, inactive conformation in the absence of Ca2+ and an extended conformation in the presence of Ca2+ or high ionic strength. Electron microscopy reveals that in the absence of Ca2+ the heads and tail are both folded to give a triangular shape, very different from the extended appearance of myosin V at high ionic strength. A recombinant myosin V heavy meromyosin fragment that is missing the distal portion of the tail domain is not regulated by calcium and has only a small change in sedimentation coefficient, which is in the opposite direction to that seen with intact myosin V. Electron microscopy shows that its heads are extended even in the absence of calcium. These data suggest that interaction between the motor and cargo binding domains may be a general mechanism for shutting down motor protein activity and thereby regulating the active movement of vesicles in cells.

Mammalian myosin Va is involved in the transport of melanosomes, the pigment granules found in melanocytes, and secretory granules in neuronal cells (1–4). Its enzymatic and mechanical properties demonstrate that it is a processive motor, capable of taking many 35-nm steps per encounter with actin filaments (5–8). The 35-nm step coincides with the half-helical repeat of the actin filament and allows myosin V to effectively walk along one side of an actin filament (9).

The myosin V heavy chain is composed of four structural domains. The N-terminal motor domain possesses the actin and nucleotide binding sites of the molecule and is followed by a neck domain containing six IQ motifs which bind calmodulin (CaM) (10). The proximal portion of the tail contains several segments of coiled-coil, driving the dimerization of two heavy chains, and the distal portion comprises a globular cargo binding domain. Electron micrographs of myosin V confirm that the molecule contains two elongated heads, a short rod and a bifurcated globular domain (11).

Tissue-purified myosin V requires micromolar Ca2+ for full MgATPase yet moves actin independent of the Ca2+ concentration in the in vitro assay (5, 11–13). Ca2+ also regulates the binding of myosin V to actin in the presence of ATP (14). However, recombinant myosin V heavy meromyosin-like fragments (HMM), which are missing the globular tail domain and the distal portion of the coiled-coil domain, have high actin-activated MgATPase rates in the absence of Ca2+ and are partially inhibited by Ca2+ if excess CaM is not present (10, 15), suggesting that the globular tail domain may be involved in down-regulation of enzymatic activity in the absence of Ca2+.

Here we show that myosin V forms a compact structure in the absence of Ca2+ wherein the tail is folded and the heads fold back against it. This occurs under conditions where the enzymatic activity is largely turned off. A model is proposed to account for the regulation of vesicle trafficking in cells.

**Experimental Procedures**

Preparation of Proteins—Myosin V was isolated from mouse brain and an HMM-like recombinant fragment of myosin V was expressed in Sf9 cells as described previously (10). The latter preparation contains a single-headed myosin V HMM molecules as seen by Conomassie Blue staining of SDS gels, coupled with Western blotting using an anti-FLAG antibody. Actin and CaM were prepared as described previously (16, 17).

Biochemical Assays—The actin-activated MgATPase activity was measured using an NADH-coupled assay (7), and the sliding actin in vitro motility assay (10) was described previously. The free Ca2+ concentration was calculated as described previously (18).

Analytical Ultracentrifugation—Sedimentation velocity runs were carried out at 20 °C and 50,000 rpm. Sedimentation boundaries were analyzed using time derivative analysis as described previously (19–21) using the recently developed software package Sedanal (22). A stock solution of myosin V or myosin V HMM was dialyzed overnight against buffer of 80 mM KCl, 20 mM MOPS (pH 7.4), 2.5 mM MgCl2, 0.1 mM EGTA, 2 mM dithiothreitol. Just before running ATP (1 mM) was added, and when appropriate, CaCl2 (0.2 mM) was added. Solutions in the range 0.1–1.0 mg/ml of protein were prepared using the dialysate as diluent. The density and viscosity calculated with Sednterp for the 80 mM KCl buffer were 1.0020 g/cc and 1.0013 cp, respectively, and for the 500 mM KCl buffer they were 1.0216 g/cc and 0.99592 cp, respectively. The value of the partial specific volumes for myosin and HMM were 0.742 and 0.741 cm3/g, respectively, calculated from the amino acid residues (23).

Electron Microscopy—Myosin V was diluted to 80 nM protein, 50 mM (or 500 mM) KCl, 20 mM MOPS (pH 7.4), 2.5 mM MgCl2, 10 μM ATP, 0.1 mM EGTA, 0.3 mM dithiothreitol, and 0.2 mM total Ca2+ when required

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The abbreviations used are: CaM, calmodulin; HMM, heavy meromyosin; MOPS, 4-morpholinopropanesulfonic acid; cp, centapoise.
Regulated Conformation of Myosin V

**Fig. 1. Effect of Ca$^{2+}$ on the actin activated MgATPase activity of myosin V.** A, titration of the MgATPase activity with increasing free Ca$^{2+}$ concentration. The conditions were 50 mM KCl, 2 mM MgCl$_2$, 1 mM ATP, 20 mM MOPS, 1 mM EGTA, 25 mM myosin V, 1 μM calmodulin, 10 μM F-actin, and varying CaCl$_2$ concentrations, 25 °C. B, actin titration of the MgATPase activity of myosin V in the presence (solid circles and left ordinate) or absence (open circles and right ordinate) of Ca$^{2+}$. Conditions were 50 mM KCl, 2 mM MgCl$_2$, 1 mM ATP, 20 mM MOPS (pH 7.0), 16 mM myosin V, 1 μM calmodulin, and either 0.1 mM EGTA or 0.1 mM EGTA and 0.15 mM CaCl$_2$, 25 °C. The $V_{max}$ and $K_{ATPase}$ were 14.7 s$^{-1}$ and 0.75 μM in the presence of Ca$^{2+}$ and 0.52 s$^{-1}$ and 0.32 μM in the absence of Ca$^{2+}$, respectively. Note the different the scales on the ordinates. The activity in the presence of Ca$^{2+}$ is on the left ordinate and that in the absence of Ca$^{2+}$ is on the right ordinate. All rates are expressed per myosin V head per second. The lines are fits to the Michaelis-Menten equation.

at 20 °C. 5 μl was immediately applied to a UV-treated carbon-filmed electron microscope grid and stained with 1% uranyl acetate as described previously (9). Micrographs were recorded and processed as described (9) except that the 14.4-nm spacing of paramyosin filaments was used for calibrations.

**RESULTS**

**Ca$^{2+}$ Activates the MgATPase Activity of Myosin V.—** Similarly to previous results (12), we find that the actin-activated MgATPase activity of purified brain myosin V is strongly activated by the presence of micromolar Ca$^{2+}$ (Fig. 1A). The lower activity in the absence of Ca$^{2+}$ is due to an effect on the maximal ATPase activity, $V_{max}$ rather than on the apparent affinity for actin, $K_{ATPase}$ (Fig. 1B). Thus, the average steady state $V_{max}$ of 12.6 ± 3.2 s$^{-1}$ with a $K_{ATPase}$ of 0.33 ± 0.2 μM in the presence of Ca$^{2+}$ and 0.47 ± 0.1 s$^{-1}$ with 0.41 ± 0.1 μM in the absence of Ca$^{2+}$. In marked contrast to intact myosin V, recombinant myosin V HMM shows a high actin-activated MgATPase even in the absence of Ca$^{2+}$, provided that 1 μM CaM is present in the assay buffer (10, 15) (Table I). Ca$^{2+}$ also increases the MgATPase activity of myosin V in the absence of actin (Table I).

**Ca$^{2+}$ Induces a Large Conformational Change in Myosin V.—** To assess whether myosin V exhibits a Ca$^{2+}$-sensitive conformational change, we measured its $S$ values in the analytical ultracentrifuge in the presence and absence of Ca$^{2+}$ under ionic conditions similar to those used for ATPase measurements. In the absence of Ca$^{2+}$, myosin V sediments with an $S$ value of 13.7 S (Fig. 2A, Table II). In the presence of Ca$^{2+}$, this value reduces to 10.7 S. We will refer to these values as 14 and 11 S, respectively. Masses estimated using the sedimentation coefficient and diffusion coefficient during the runs show that the molecule is monomeric under both conditions. The shift in $S$ value is consistent with myosin V adopting a more compact structure in the absence of Ca$^{2+}$, conditions where the ATPase is low, and changing to an extended structure in the presence of Ca$^{2+}$, conditions where the ATPase is high.

Under ionic conditions identical to those used for tissue purified myosin V, HMM underwent a smaller change from 8.5 S in the absence of Ca$^{2+}$ to 9.4 S in its presence (Fig. 2B, Table II). Note that the direction of this change is opposite to that for intact myosin V and is consistent with HMM being more compact in the presence of Ca$^{2+}$ than in its absence. Inclusion of 2 μM exogenous CaM in the buffer did not affect the sedimentation values for the HMM in either the presence or absence of Ca$^{2+}$ suggesting that the effect is not merely due to dissociation of CaM in the presence of Ca$^{2+}$ (Table II).

**A Compact Structural State for Myosin V—** Myosin V was viewed in the electron microscope following negative staining. In the absence of Ca$^{2+}$ (at 80 mM KCl), the majority of the molecules on the grid showed a novel, compact triangular shape (Fig. 3a). Single particle image processing reveals an enigmatic but rather consistent substructure (Fig. 3b) in which both the 32-nm heads and 57-nm tail must be folded because the lengths of the sides of the triangle (22–27 nm in the image averages) are shorter than either of these domains (compare

| [KCl]  | MgATPase activity | Actomyosin |
|--------|------------------|------------|
|        | Ca$^{2+}$ alone | +Ca$^{2+}$ |
| 50 mM  | 0.029 s$^{-1}$   | 0.080 s$^{-1}$  |
| 500 mM | 0.063 s$^{-1}$   | 0.064 s$^{-1}$  |
|        | 2.20 s$^{-1}$    | 1.33 s$^{-1}$   |

**TABLE II**

| Sedimentation coefficients (s$_{wp}$) |
|-----------------|--------|--------|
| [KCl] | Ca$^{2+}$ | EGTA |
| Myosin V | 80 mM | 10.7 | 13.7 |
| Myosin V | 500 mM | 9.8 | 9.6 |
| HMM | 80 mM | 9.4 | 8.5 |
| HMM + CaM | 80 mM | 9.4 | 8.5 |
When purified myosin V or HMM was viewed at low ionic strength in the presence of Ca\(^{2+}\), few identifiable structures were observed whether or not 1 mM exogenous CaM was present (Fig. 3c). We believe this is due to either full or partial dissociation of individual CaM light chains from their IQ motif binding sites which would allow the levers to be more flexible and adopt very diverse shapes. Myosin II light chain dissociation has previously been shown to produce a more compact lever arm (24). In the presence of 1 mM exogenous CaM, elongation of the Ca\(^{2+}\) by 5 mM EGTA reverses the instability of myosin V over a period of 30 min so that the compact appearance is restored, and on subsequent addition of salt the extended conformation is also seen.

**DISCUSSION**

We have shown that myosin V adopts a compact folded conformation with a sedimentation coefficient of 14 S under *in vitro* conditions where the actin-activated MgATPase activity is turned off. The reason this structure has not been seen before is that the high ionic strength conditions used in earlier microscopy favor the extended form. Several interesting questions arise. First, how does myosin V translocate cargo in cells where the Ca\(^{2+}\) levels are typically less than one micromolar, and second, how is the lack of regulation seen in *in vitro* motility assays reconciled with the tight regulation observed in MgATPase assays? These questions may be linked. We postulate that the binding of myosin V to its docker or linker protein unlocks the folded conformation and results in an active myosin even in the absence of Ca\(^{2+}\). For example, myosin V in mammalian melanocytes binds via its tail domain to the C terminus of melanophilin, which, in turn, binds via its N terminus in a GTP-dependent manner to Rab27a to make an active tripartite complex (25–27). The tail of myosin V is alternatively spliced and the melanocyte specific isoform has an exon required for binding of melanophilin that is not present in the brain myosin V isoform we isolated in this study (25). We predict that addition of melanophilin to the melanocyte-specific isoform but not to the brain isoform triggers the extended active conformation even in the absence of Ca\(^{2+}\). It is likely that other myosin V isoforms have similar linker proteins that would act in an analogous manner. It is possible that binding of myosin V to a coverslip surface, *in vitro*, triggers the same conformational change in myosin V, leading to an active molecule. This would explain why myosin V is always active in assays where it is bound to a surface.

An advantage of having a highly regulated myosin V is that unregulated, non-cargo-bound myosin V in cells would needlessly hydrolyze ATP and would stay associated with actin filaments due to its processivity. This behavior would be even more pronounced in elongated cells such as neurons and melanocytes where myosin V participates in the transport of vesicles to the dendritic tips for docking with the cell membrane. If the activity of the molecule were unregulated myosin V would not be available for newly formed vesicles or cargo in the cell body. If, on the other hand, myosin V could dissociate from the vesicle after docking and fold into an inactive form that could freely diffuse, it could then be effectively recycled.

We propose a model whereby free myosin V in cells under conditions of low Ca\(^{2+}\) concentration exists in the 14 S conformation and is enzymatically and mechanically inactive (Fig. 4). The binding of cargo to the tail region occurs either directly or indirectly via linker proteins and triggers myosin V to adopt the extended, active conformation. This interaction might be indirectly via linker proteins and triggers myosin V to adopt the extended, active conformation. This interaction might be regulated by phosphorylation of myosin V or any of the linker proteins or by the nucleotide status of a G-protein that is part of the linker complex. The change to the extended conformation is also triggered directly by Ca\(^{2+}\) binding to myosin V, but this
Regulated Conformation of Myosin V

The next challenges with myosin V will be to identify the putative interacting portions of the head and tail and to determine how these interactions are regulated and how they are disrupted by cargo binding.

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FIG. 4. Speculative model for conformation-dependent regulation. Under normal low Ca2+ conditions in cells, myosin V would be either bound to cargo or folded into an inactive form that would not move on actin filaments. Regulation of cargo binding to myosin V either directly or via linker proteins might be regulated by the state of phosphorylation of the myosin V cargo binding domain, phosphorylation of the linker protein, or by the nucleotide state of G-proteins. Increases in cellular Ca2+ levels might elevate the actin-activated MgATPase activity, but if CaM molecules dissociate, little mechanical activity would be produced. The schematic is not to scale and the depiction of the 14 S conformation is speculative.

may result in a mechanically weakened protein, since some CaM molecules dissociate from myosin V in the presence of Ca2+ (15, 28). Myosin II stripped of one of its light chains retains its actin-activated MgATPase activity but performs poorly in mechanical assays such as the in vitro motility assay or step size measurements using the optical trap (29–31). Thus, even though myosin V adopts the 11 S extended conformation in the presence of Ca2+, it may not be a fully functional motor under these conditions.

Regulation of motor protein activity by a tail-dependent conformational change plays a role in the regulation of conventional kinesin (32–34) and has been adopted by at least two myosin family members (II and V). In addition, there is indirect evidence for a role of the tail in the regulation of a mammalian myosin I (35). The structural basis for the regulation of myosin V is very different from that of smooth muscle myosin II, however. The enzymatic activities of smooth and nonmuscle HMM are regulated by phosphorylation even though these molecules are missing much of their tails, whereas that of myosin II HMM is unregulated (36, 37). The compact folded structure observed with smooth and nonmuscle myosins may be more important for regulation of filament assembly than for enzymatic activity. A particular asymmetric interaction between the two heads of smooth muscle HMM is seen in the off state that may be important for the enzymatic regulation (38). The compact form of myosin V also has a specific structure, but it differs in having the globular tail domains incorporated within the head region. The fact that the microtubule-dependent motor, kinesin, and two myosin family proteins are now known to have evolved regulatory mechanisms involving head-tail interactions that occur via large conformational changes is very significant. It suggests that future studies testing for motor molecule regulation should examine the enzymatic properties of complete molecules, along with any associated subunits. In kinesin the portion of the globular tail domain responsible for the inhibition has been identified (33).