Walking with myosin V

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The cytoplasm of cells is teeming with vesicles and other cargo that are moving along tracks of microtubules or actin filaments, powered by myosins, kinesins and dyneins. Myosin V has been implicated in several types of intracellular transport. The mechanism by which myosin V moves processively along actin filaments has been the subject of many biophysical and biochemical studies and a consensus is starting to emerge about how this minute molecular motor operates.

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Introduction

Intracellular motility of vesicles is driven by molecular motors that move along microtubules or actin filaments. The best-characterized actin-dependent motor is myosin V (myoV). Mouse myoV has two motor domains connected to extended necks with six bound calmodulins dimerized by a coiled-coil α-helix (Figure 1). The molecule ends in a globular tail domain. Organelle motors are often processive in that they can take tens to hundreds of steps along actin filaments before dissociation, thus ensuring efficient translocation of their cargo over micrometer distances. MyoV has evolved four molecular properties that enable it to function efficiently as an organelle motor. First, its globular tail domain binds to cargo via adapter protein complexes [1–3]. Second, the tail domain self-associates through a coiled-coil domain to form a two-headed dimer. Third, its biochemical cycle time is dominated by states with a high affinity for actin; this favors the attachment of at least one of the two heads at all times, preventing the cargo from dissociating prematurely from the actin filament between kinetic cycles [4]. Fourth, its long neck allows the molecule to take 36-nm steps, a length equal to the pseudo-repeat distance of the helical

actin filament. Thus, myoV steps more or less straight along the longitudinal axes of the actin filament with the cargo remaining above the cytoskeleton, which reduces viscous drag and eliminates the necessity of spiraling around the actin filament. The structural adaptations are shown schematically in Figure 1.

The 36-nm step size is observed directly in single molecule mechanical [5–8,9••,10,11] and fluorescence studies [12,13•,14,15••,16••] and is consistent with electron microscopic images of myoV molecules bound with both heads to actin in the presence of ATP [17,18]. Many of these studies support the idea that a 36-nm step is produced by a combination of a working stroke, where a single bound head rotates its neck region to move the centre of mass of the molecule forward by ∼20–25 nm, and a thermal component, where the free head undergoes a diffusional search for a new binding site, which moves the centre of mass forward by another ∼11–16 nm [5,10,17,18,19••,20,21]. Electron micrographs of myoV molecules attached by only a single head suggest that after the power-stroke has occurred the free head becomes positioned to explore the binding sites in forward direction along the actin filament [17]. To account for the narrow distribution of the step sizes seen in single-molecule mechanical experiments and the relatively constant distance between the two heads when both are bound (13 actin monomers) observed in the EM images, we have to assume that myoV has a great preference for binding to the actin monomer which provides least azimuthal distortion when both heads are bound.

The key to processivity lies in the kinetics

The most salient feature of myoV's kinetic cycle is the rate-limiting release of ADP from AM.ADP, a kinetic intermediate where actin and myosin bind with high affinity [4]. Actin-activated phosphate release is fast [4], unlike in the conventional myosin II class proteins where this step is rate-limiting. Studies in which nucleotide-free myoV crystals were soaked with MgADP showed that ADP was bound whereas the Mg ion was not [22•], suggesting that in the ATPase cycle Mg^{2+} is released prior to ADP. This prediction is supported by kinetic studies [23,24] and suggests that free Mg^{2+} concentration may modulate myoV's kinetics. MyoV binds more rapidly to actin in the absence of nucleotide than do other myosin isoforms, possibly because the cleft separating the upper and lower 50-kDa domains of myoV is partially closed even when myosin is detached from actin [25]. The actin affinity of the so-called weakly bound states — AM.ATP and AM.ADP.Pi — is stronger in

Figure 1
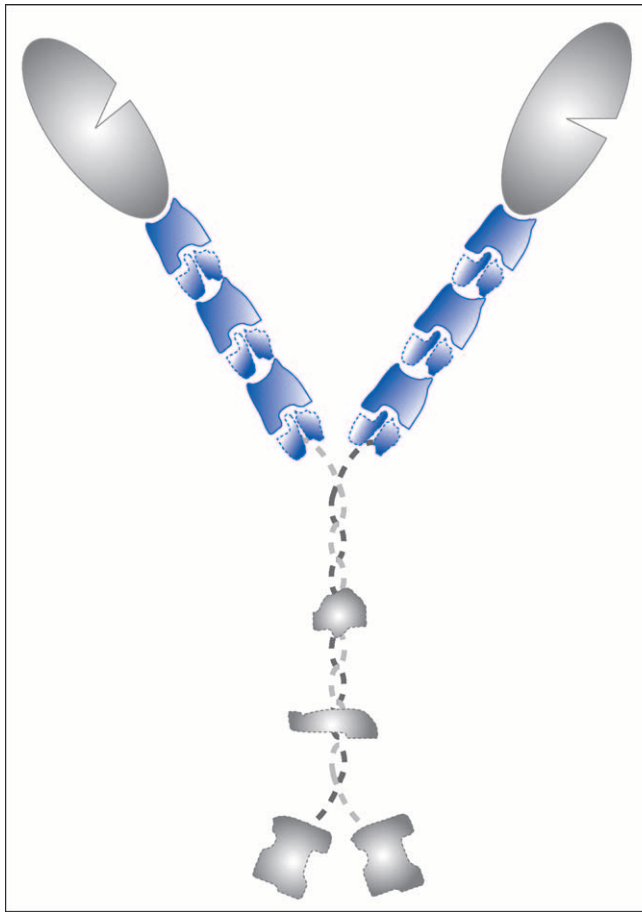
Schematic diagram of mouse myosin V. The myosin heavy chain is depicted in various shades of grey. Calmodulin molecules are shown in blue.

myoV than in most other myosins, and this affinity is modulated by charged residues in an actin-binding surface loop [26,27]. Using the rate constants determined in kinetic studies of the single-headed fragment, it was estimated that myoV spends ~70% of its kinetic cycle strongly bound to actin; it is therefore said to have a 'high duty cycle' [4].

The solution kinetic parameters of a single head are consistent with the kinetics of stepping seen in the mechanical [5–8,9••] and fluorescence studies [12,13•, 14,28], suggesting that ADP release is also rate-limiting during processive movement of the dimeric motor. The stall force for myoV is ~3 pN [7]. Beyond this force, myoV often detaches from actin and then reattaches to begin a new run. The run lengths for myoV show little load dependence, at least for opposing and assisting loads in the range of ±2 pN [9••]. Physiologically, this might enable myoV to transport cargo efficiently and robustly against variable drag forces through the actin cytoskele-

ton. Simulations of the processive movement suggest that, as a result of the elastic coiled-coil connection between a bulky cargo and myoV, the motor does not have to generate forces large or prolonged enough to make the cargo follow the motors movements immediately. The compliant link can transiently absorb the abrupt mechanical transitions of the motor molecule and, interestingly, impose a highly regular gait on the motor [29•]. Related simulations link the cargo size and connection compliance to the stepping velocity of the motor [30]. For forces close to stall force (i.e. 2–3 pN), dwell times at saturating ATP become longer with increasing force and backward steps are seen with increasing frequency [5–8,9••]. This suggests that there are load-dependent steps in the kinetic cycle, possibly associated with ADP release — or an isomerization preceding ADP release — on the leading and/or trailing head [5,7,12], or a load-dependent diffusive component associated with the lead head searching out the next binding position along the actin filament [5–8,9••,19••,20,31•]. At opposing forces beyond stall (5 pN) the motor produces processive backward steps of 36 nm [9••]. However, it is unclear in which way backward steps and their kinetics are related to the biochemical cycle of the heads.

Kinetic gating increases the run length

Given that native myoV has two heads, each with a duty ratio of 0.7, only about eight processive steps per diffusional encounter with actin are expected, assuming no cooperativity between the two heads [5]. *In vitro*, single molecule studies reveal myoV to be much more processive than this, taking on average ~10–60 steps [8,9••,10,12,13•,14,28,32••,33]. The key to this increased processivity seems to lie in a strain-dependent kinetic gating mechanism of the two heads. At low load and saturating ATP, the dwell times for the interactions of a single myoV head are two- to three-fold longer than the dwell times between steps of double-headed molecules during a processive run [5,21], suggesting that the action of the leading head on the trailing head increases the rate of ADP release from the latter. Such a load-dependent gating mechanism could serve to keep the biochemical cycles of both heads out of synchrony and increase the processive run length by reducing the probability of both heads detaching from actin during the same time period. Biochemical evidence for head–head gating came from experiments which resolved the biphasic kinetics of ADP release for a dimeric myoV construct in the presence of actin when both heads were strained by simultaneously binding to an actin filament [34••]. The data were consistent with a model in which ADP release is accelerated two- to three-fold on the trailing head and slowed down -50-fold on the leading head, as compared to ADP release from a single-headed species.

Direct measurements of the load dependence of acto-myosin interactions were recently performed using

single-molecule mechanical techniques. In one study the effect of load on the attached lifetimes of a single myoV head (S1) was measured over a range of loads over ±2 pN [19$^{\bullet\bullet}$]. Analysis of the time course of single acto-S1 inter-actions revealed that, for each ATP hydrolyzed, a working stroke in two sub-steps (∼16 nm + ∼5 nm) is produced, probably coupled to Pi and ADP release or isomerization preceding or following product release [5]. This is sup-ported by recent cryo-EM data comparing acto-myoV complexes in different nucleotide states [35$^{\bullet\bullet}$]. The first sub-step occurs within 3 ms of myosin binding and is followed by an ATP-independent but load-dependent delay, probably consisting of some ADP state(s) and ter-minated by the second ∼5-nm sub-step. This is followed by an ATP-dependent phase that is only slightly affected by load and probably represents the nucleotide-free rigor state. This phase is terminated by ATP binding and detachment. Another study, in which the effect of two different loads on the total attached lifetime was compared, confirmed shorter lifetimes for a pushing and longer ones for a pulling force of 2 pN in these experiments. Here, the effect of ATP and ADP concentrations on the lifetimes was also consistent, with a load-dependent ADP state that seems less sensitive for a pushing than for a pulling force [31$^{\bullet}$]. Intramolecular strain, imposing force in opposite directions on the heads, is expected to arise as a result of the disparity between the 20–25-nm working stroke of a single head and the 36-nm distance between the heads when both are bound. EM images of myoV bound on actin via two heads support the idea of intramolecular strain [17,18].

MyoV moves via a hand-over-hand mechanism where the neck region acts as a rigid leverarm

Two variations of TIRF microscopy provide evidence that myoV moves in a hand-over-hand mechanism whereby the two heads alternate leading and trailing positions. Recently, a fluorescence technique termed FIONA (fluorescent imaging with one nanometer accu-racy) was introduced [12] to monitor processive move-ments of single, fluorescently labeled myoV molecules on immobilized actin filaments under unloaded conditions [12,13$^{\bullet}$,14,15$^{\bullet\bullet}$,16$^{\bullet\bullet}$]. If sufficient photons are collected, the point spread function, and thus the mean position of immobilized fluorophores, can be determined at 1–2-nm resolution [12]. MyoV molecules with a single cy3-labeled calmodulin [12,13$^{\bullet}$,14] or GFP fused to the N terminus of one head [14] produced step sizes and kinetics consistent with a hand-over-hand mechanism where the rear head moves forward the distance of one actin repeat (72 nm) as it becomes the new leading head. This distance is con-sistent with the step size of 36nm found in the mechanical experiments measuring the movement of the center of mass of the molecule. Even more direct evidence for a hand-over-hand mechanism came from FIONA studies in which the two heads were differentially labeled with

fluorophores of different colors, which allowed visualiza-tion of the alternating movements (72 nm) of the two heads and showed a constant 36-nm separation between the two attached heads [15$^{\bullet\bullet}$,16$^{\bullet\bullet}$]. In other experiments, fluorescence polarization was used to determine the angles assumed by a fluorescent probe bound to calmo-dulin in the neck region of myoV. Alternating angles were observed while myoV was moving processively that were consistent kinetically and structurally with a model in which the two heads are moving alternately in a hand-over-hand mechanism [28].

The notion that the light-chain binding region or neck of myosin might function as a lever arm by making a large-angle rigid-body motion about a pivot point in the motor domain was originally suggested by myosin II crystal structures, which showed various positions of the neck region in different nucleotide-bound states (see [36] for review). Negatively stained EM images of myoV in the absence of actin showed nucleotide-dependent leverarm swings [18], and myoV leverarm swings were directly observed by fast AFM imaging of myoV in the presence of nucleotide [37]. MyoV crystallography and a combi-nation of cryo-EM of myosin-bound actin filaments with the docking of crystal structures into three-dimensional reconstructions suggest that small movements of motor domain elements that occur in response to nucleotide binding and hydrolysis are transmitted through a trans-duction pathway to allow the neck region of myoV to swing as much as ∼75–105°, which would correspond to a displacement of the tip of the lever arm of 20–36 nm [17,22$^{\bullet}$,35$^{\bullet\bullet}$]. Very strong support for the lever arm model has come from mechanical experiments where the neck region of myoV has been either elongated or truncated by the addition or subtraction of IQ motifs [10,13$^{\bullet}$,20,38]. In these studies it was shown that the speed of actin filament displacement over immobilized myosin heads, the speed of single myoV molecules moving along immobilized actin filaments, the size of the power-stroke produced by a single myoV head, and the step-size in processive runs of dimeric myoV all varied roughly linearly with the length of the myoV lever arm.

The question of whether myoV requires exactly six IQ motifs to be highly processive has also been addressed. It is clear that myoV molecules with two, four, six or eight IQ motifs could all move processively [10,13$^{\bullet}$,20,31$^{\bullet}$]. Given that molecules with fewer or more than six IQ motifs take steps that are shorter or longer than 36 nm, it is likely that these molecules spiral around the actin filament while moving. A two-headed myoV fragment with only one IQ motif did not move processively [10], but another study found that a chimera containing the myoV head with one IQ motif fused to the rod domain of smooth muscle myosin could take two to three successive 36-nm steps [11].

All of the detailed biochemical and kinetic experiments described above were performed using mouse or chicken myoVa, but processivity is not a universal feature of class-V myosins. Transient kinetic analysis of *Drosophila* myoV suggests that ADP release is not rate limiting here and the molecule is not capable of moving actin filaments in a processive manner in motility assays [39]. In addition, it was concluded on the basis of actin landing assays that the two type-V myosins from *S. cerevisiae*, myo2p and myo4p, are not processive either [40]. It should be noted, however, that these difficult assays are an indirect measure of processivity and more direct assays such as optical trapping or TIRF motility assays have not yet been carried out on the yeast myosins.

Models for processive movements

Several models have been presented for the processive movement of myoV. Nearly all assume that the two-headed structure is necessary (for an alternative, see [41]). The critical issues are the nucleotide state and the

actin affinity of the two heads of myosin during dwell periods (Figure 2). Optical trapping studies, measuring the stiffness of acto-myosin during attachment, detected a reduced level of stiffness just prior to each step transition, suggesting that, at saturating ATP concentrations and low load, the molecule dwells mostly with both heads strongly bound to actin [states (b) or (c) in Figure 2] [5]. This is consistent with the biochemical, mechanical and structural studies suggesting that, under these conditions, myoV dwells with both heads strongly bound in some ADP state(s) and that the detachment kinetics of the heads are determined by a slightly accelerated and strongly reduced ADP release on the trail and lead heads, respectively, due to intramolecular strain [5,6,19••,31•,34••]. In this model myoV moves processively as long as the lead head rebinds strongly to actin before the trail head releases ADP and subsequently binds ATP, resulting in detachment of that head and, thus, the whole molecule. The pathway in Figure 2 would be (a)→(b)→(c)→(d) for a single step. Unresolved issues are whether the lead head

Figure 2
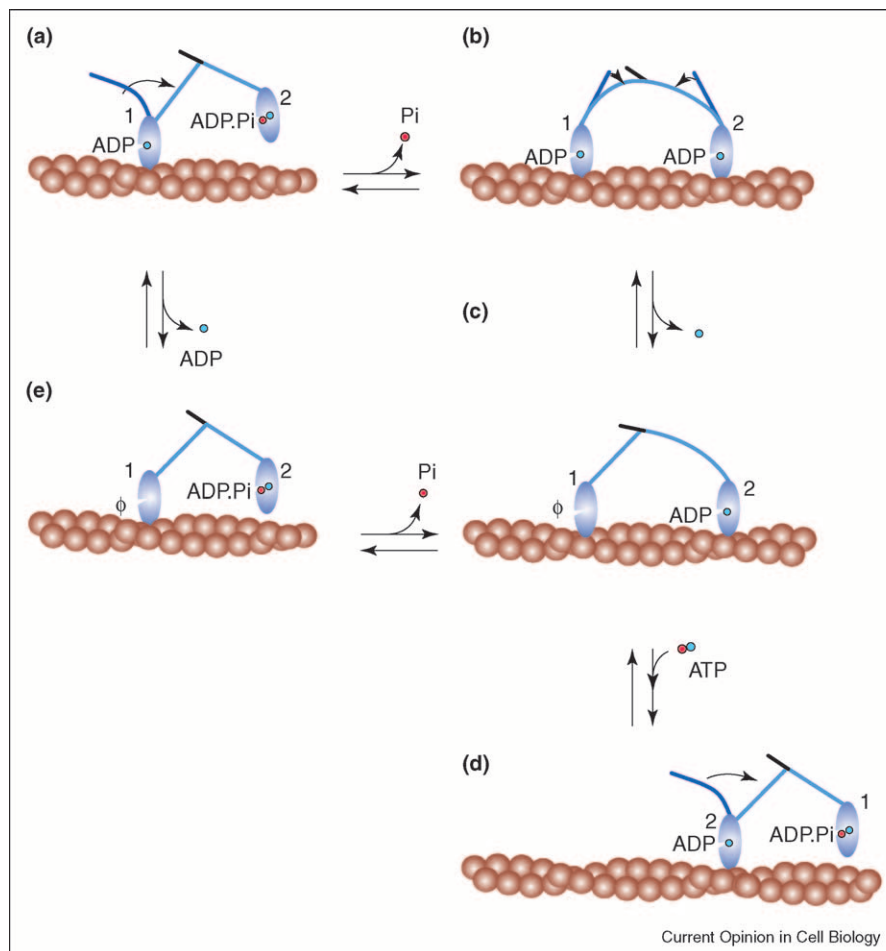
Models for processive stepping. Two main pathways — (a)→(b)→(c)→(d) or (a)→(e)→(c)→(d) — are indicated as described in the text. The red dot represents Pi and the blue dot represents ADP. The faint blue lines in (c) represent a case where the lead head has gone through its working stroke and increased the intramolecular strain.

can produce its working stroke while the trail head is still bound, and the coupling between conformational changes and ADP, Pi release or isomerization processes.

In contrast, a model derived from fitting the nucleotide dependence of run length and velocity of myoV suggests two differences from the above model. First, the dwell state is dominated by a molecule with ADP in the strongly bound rear head and ADP.Pi in the weakly bound lead head [state (a) in Figure 2]; second, termination occurs from this same state [32**]. Starting with state (a), two stepping pathways are proposed. In the first, the lead head attaches, releasing its phosphate and resulting in two ADP-bound heads attached to actin [state (b)], like in the model above. In the second, the trailing head releases ADP while the lead head is still weakly bound [state (e)]. The limiting step in these two pathways would be the attachment of the lead head, coupled to Pi release from that head [(a)→(b)] or ADP release from the attached, trailing head [(a)→(e)]. Termination of processive runs would most commonly occur via dissociation from state (a) in Figure 2. Kinetic studies of two-headed chicken myoVa however suggest that phosphate release is fast, which would appear to disagree with the above model, but the effect of differences in species and assay conditions must be explored [34**].

Conclusions

MyoV is an ideal molecule to dissect the molecular basis for processivity. It expresses well in *in vitro* systems, so mutants can be prepared and studied. It is large enough to be easily seen in electron microscopic images and has a high affinity for actin. Its kinetic and many of its mechanical properties have been elucidated in a series of elegant experiments. This has provided important information to test stochastic models interpreting measured force–velocity relationships and relating those to the kinetics and substeps of load-dependent forward and backward reactions [42]. In order to test models addressing head coordination and stepsize distributions in myoV [43,44], additional stiffness measurements of the dimeric motor and of a single myosin head in different biochemical states are required and will provide complementary information to structural studies on the myoV light chain binding domain, which is assumed by many to act as a leverarm [45]. The next advances will require the combination of mechanical studies with single molecule fluorescence measurements reporting nucleotide binding. An understanding of the molecular mechanism for myoV action will contribute to an understanding of force generation in other myosins and in other molecular motors.

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