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# The Yeast Class V Myosins, Myo2p and Myo4p, Are Nonprocessive Actin-based Motors

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***Abstract.*** The motor properties of the two yeast class V myosins, Myo2p and Myo4p, were examined using in vitro motility assays. Both myosins are active motors with maximum velocities of 4.5 μm/s for Myo2p and 1.1 μm/s for Myo4p. Myo2p motility is $Ca^{2+}$ insensitive. Both myosins have properties of a nonprocessive motor, unlike chick myosin-Va (M5a), which behaves as a processive motor when assayed under identical conditions. Additional support for the idea that Myo2p is a nonprocessive motor comes from actin cosedimentation assays, which show that Myo2p has a low affinity for F-actin in the presence of ATP and $Ca^{2+}$, unlike chick brain M5a. These studies suggest that if Myo2p functions in organelle transport, at least five molecules of Myo2p must be present per organelle to promote directed movement.

Key words: myosin-V • cytoskeleton • in vitro motility • processive motor • *cerevisiae*

## *Introduction*

The class V myosins represent one of 18 distinct classes of actin-based molecular motors (Berg et al., 2001). Class V myosins, which have been well-characterized in several organisms, including yeast, chicken, mouse, and squid, have been shown to play a role in both membrane trafficking and RNA transport (for review see Reck-Peterson et al., 2000). Consistent with many of the proposed functions for class V myosins, such as movement of organelles, biophysical studies of the class V myosins have revealed that myosin-Va (M5a)[1] purified from chick brain is the first example of a processive actin-based motor (Mehta et al., 1999; Rief et al., 2000; Sakamoto et al., 2000). Processive motors, like the microtubule motor conventional kinesin, are capable of coupling multiple rounds of ATP hydrolysis to movement, such that single motor molecules can move their cargo long distances along a given filament without diffusing away from it (Howard, 1997). In the case of M5a, this motor takes 36-nm steps, equivalent to the helical repeat of the actin filament, indicating that this motor can walk along a single filament without spiraling around it (Mehta et al., 1999; Rief et al., 2000; Walker et al., 2000).

The yeast, *Saccharomyces cerevisiae*, has two class V myosins, Myo2p and Myo4p (for review see Reck-Peterson et al., 2000). Myo2p and Myo4p are 71% identical in their motor domains, whereas only 18% similar in their globular tail domains (Reck-Peterson et al., 2000). Myo2p and Myo4p appear to have distinct and nonoverlapping functions in yeast cells, perhaps due to the low level of similarity between the tail domains of these two myosins. Both Myo2p and Myo4p are thought to promote their hypothesized functions by directly transporting cargo to polarized regions of the cell. Myo4p has been proposed to transport an mRNA needed to repress mating type switching in daughter cells (Long et al., 1997; Takizawa et al., 1997), whereas Myo2p has been hypothesized to transport secretory vesicles, the vacuole and components involved in mitotic spindle orientation (Reck-Peterson et al., 2000; Yin et al., 2000).

The proposed roles for the yeast class V myosins require that these motors act as processive motors or use multiple motors in concert to promote continuous cargo movement. To determine whether processivity is a general property of class V myosins, we have investigated the motor properties of the two yeast class V myosins, Myo2p and Myo4p, using

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[1]*Abbreviations used in this paper:* CaM, calmodulin; M5a, myosin-Va.

a modification of the sliding filament in vitro motility assay (Post et al., 1998). Our results show that both Myo2p and Myo4p are nonprocessive class V myosins.

## Materials and Methods

### Yeast Strains, Growth, and Lysis Conditions

The yeast strains used in this study are listed in Table I. Yeast cells were grown in YP medium containing 2% dextrose at 30°C. The *MYO4*-GFP strain (Karpova et al., 2000) has not been tested for mating type switching defects. However, since the GFP tag is at the COOH terminus, it would not be expected to affect motor function.

Log phase cells from 100 ml of NY580 or NY1125 (Myo2p assays) were lysed in buffer A (20 mM imidazole, 75 mM KCl, 1 mM EGTA, 2.5 mM $MgCl_2$, 2 mM DTT, 1 mM Pefabloc-sc [Boehringer]), 10 mM pepstatin A, 10 mM leupeptin, 1 mM benzamidine, pH 7.2). Strains used for Myo4p assays (YJC1411 or YJC1777) were lysed in buffer A with additional 75 mM KCl, 5 mM Mg-ATP and 2 mM EDTA. Cells were lysed using 0.5-mm zirconium–silica beads (Biospec) using a Mini-BeadBeater-8 (Biospec) for 3 × 45 s at 4°C. The lysate was spun at 2,000 *g* for 5 min, and the resulting supernate was spun at 257,000 *g* for 20 min. The supernate (S3) from this spin was used for cosedimentation and motility assays.

### Actin Cosedimentation Assays

Actin cosedimentation assays were performed with Myo2p in S3 isolated from NY580 or NY1125 cells with or without added (4 μg/ml) purified chick brain M5a (Cheney, 1998). S3 was mixed with 7 μM chicken skeletal muscle F-actin (Spudich and Watt, 1971) in buffer A ± 4 mM ATP. To monitor the effects of $Ca^{2+}$, either 50 μM free $Ca^{2+}$ or 4 mM EGTA was added. After incubation (10 min 4°C), samples were centrifuged at 175,000 *g* for 30 min to pellet F-actin. The resulting supernate and pellet fractions were separated by SDS-PAGE. The lower half of these gels were stained with Coomassie blue to visualize F-actin, and the top half was immunoblotted with Myo2p tail antibody.

### In Vitro Motility Assays

Motility chambers (4 × 22 mm) were made on nitrocellulose-coated coverslips as described previously (Post et al., 1998). Protein A (0.5 mg/ml; Sigma-Aldrich) in 10 mM imidazole was adsorbed to the chamber surfaces for 30 min. Chambers were washed 3× with 1.0 ml TBS. Next, antibody was adsorbed to the surface for 1 h. For Myo2p, total IgG concentration was kept constant at 50 μg/ml by mixing anti-Myo2p tail (2.5–50 μg/ml; Reck-Peterson et al., 1999) with rabbit IgG (Jackson ImmunoResearch Laboratories). For Myo4p, the IgG concentration was kept constant at 100 μg/ml and, rabbit anti-GFP (gift of S. Ferro-Novick, Yale University, New Haven, CT) was used at 10–100 μg/ml. For chick M5a, the total IgG concentration was kept constant at 50 μg/ml and anti-M5a tail (Suter et al., 2000) was used at 5–50 μg/ml. After antibody absorption, the chambers were washed again 3× with TBS, blocked (30 min) with 5.0 mg/ml BSA, washed with 2 × 1.0 ml TBS and 1.0 ml buffer A, and then S3 was added to the chambers for 1–3 h. For the chicken M5a studies, purified M5a (4.0 μg/ml final concentration) was added to S3. Before viewing, chambers were washed 3 × 1.0 ml in buffer A and then 200 μl of 2nM phalloidin-stabilized F-actin in motility buffer (buffer A with 8.0 mM DTT, 200 μg/ml glucose oxidase, 0.1 mg/ml glucose catalase, 2.5 mg/ml glucose and 10 mM $Mg^{2+}$-ATP). Finally, 2nM BODIPY-TRX phallacidin-stabilized (Molecular Probes) F-actin in motility buffer was added to the chambers, and filament movements were recorded within the first 10 min.

Myo2p protein concentrations were quantified by quantitative immunoblot analysis of Myo2p content in the unbound lysate from each motility chamber using bacterially expressed Myo2p tail protein (Reck-Peterson et al., 1999) as standard. Blots were scanned, and the integrated optical density of each band was quantified using Metamorph software (Universal Imaging Corp.)

### Video Microscopy and Data Analysis

Microscopy and data analysis were performed as described (Post et al., 1998), with images recorded every 1–4 s depending on filament velocity. Velocities were determined using Metamorph Track Points (Universal Imaging Corp.) with data points weighted by the SEM. The number of filaments that landed and moved per a given time increment and field size was recorded. The errors associated with landing rates were derived from the counting statistics (mean landing rate/square root of $n$, where $n$ = number of landing events observed; Mehta et al., 1999).

The model used to determine duty ratio is a modification of that developed by Uyeda et al., (1990). Here we fit velocity as a function of motor density as follows:

$$V(\rho) = V_{max}[1 - (1 - f)^{\rho * A}]. \quad (1)$$

In this expression, $V$ is average filament velocity; $\rho$ is motor density; $V_{max}$ is maximal filament velocity; $A$ is the mean area of interaction surrounding an actin filament (approximately the product of twice the motor reach and mean filament length); and $f$ is the duty ratio. For the purposes of the theoretical curves shown in Fig. 4, $V_{max}$ = 4.7 μm/s (from the best fit to the data) and $A$ = 0.08 $\mu m^2$ (derived from the landing rate fits, see below).

To obtain an estimate of the number of motors required to bind and move a filament, the following model, originally described by Hancock and Howard (1998), was used to fit landing rate data with respect to motor density:

$$L(\rho) = Z(1 - \exp^{-\rho * A})^n. \quad (2)$$

Here, $L$ is the landing rate as a function of motor density (ρ); $Z$ is the maximum landing rate; $A$ is the average area of interaction surrounding a filament (as defined above); and $n$ is the number of motors required to bind a filament and produce continuous motion. Values obtained from the best fit to the data ($Z$ = 46.9 $s^{-1} \cdot mm^{-2}$ and $A$ = 0.08 $\mu m^2$) were fixed along with a given value for $n$ to produce the theoretical curves shown in Fig. 4.

*Table I. Yeast Strains Used in This Study*

| Name | Genotype | Source |
|---|---|---|
| NY580 | *MATα PEP4::URA3 ura3-52 leu2,3-112* | P. Novick |
| NY1125 | *MATα myo2-66 PEP4::URA3 ura3-52 his4-619* | P. Novick |
| YJC1411 | *MATα leu ura3 his3-Δ200* | J. Cooper* |
| YJC1777 | *MATα MYO4-GFP-HIS3 leu ura3 his3-Δ200* | J. Cooper |

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## Results and Discussion

### Myo2p Does Not Have a High Affinity for Actin in the Presence of ATP

Unlike the nonprocessive myosins, myosin-I and –II and chick and mouse M5a have a high affinity for actin in the presence of ATP (Nascimento et al., 1996; De La Cruz et al., 1999; Wang et al., 2000). In contrast to the chicken M5a added to S3, Myo2p cosedimented with F-actin only in the absence, but not in the presence, of 4 mM ATP (Fig. 1). Moreover, $Ca^{2+}$, which enhances the binding of chicken M5a to actin, had no effect on Myo2p binding. Myo2-66p from *myo2-66* cells showed impaired binding to actin in the absence of ATP, consistent with the presence of a point mutation in the predicted actin-binding face of the motor domain in this mutant. (Reck-Peterson et al., 2000) (Fig 1).

### Myo2p and Myo4p Are Active Myosin Motors

To investigate the motor properties of Myo2p and Myo4p, we used an antibody-based version of the sliding filament in vitro motility assay (Post et al., 1998). In chambers containing immunoadsorbed Myo2p, actin filaments were observed moving at maximum speeds of 4.5 μm/s, although some preparations exhibited somewhat slower maximum velocities (2.5–3.5 μm/s). No motility was observed when

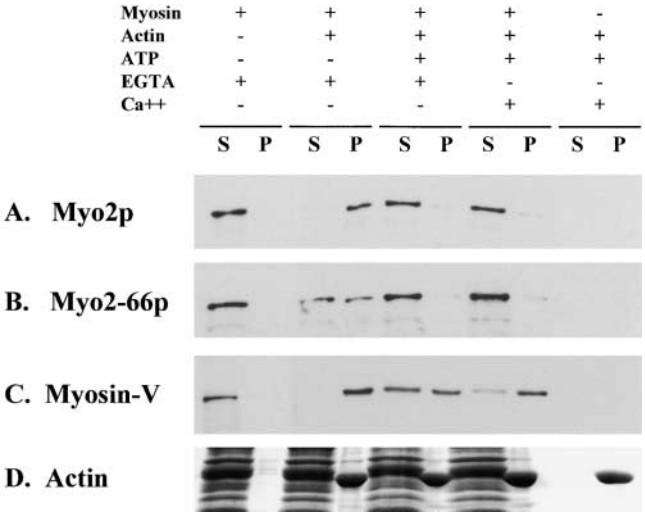


*Figure 1.* Actin cosedimentation assays. Myo2p (A), Myo2-66p (B), or chick brain M5a (C) in S3 supernates was incubated with F-actin in the presence or absence of 4.0 mM ATP with or without 50 μM free $Ca^{2+}$. After centrifugation to pellet, the F-actin, the supernate, and pellet fractions were immunoblotted with antibodies to Myo2p (A and B) or M5a (C). D shows a representative Coomassie blue–stained gel to visualize actin. In contrast to M5a, Myo2p fails to bind to actin in the presence of ATP. The motor mutant Myo2-66p exhibits reduced actin binding activity.

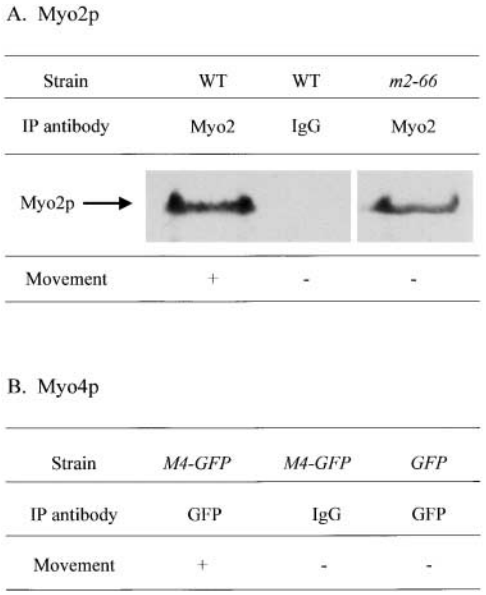


*Figure 2.* Motility of Myo2p, Myo2-66p, and Myo4p. (A) Immunoblot analysis of antibody capture motility chambers containing either anti Myo-2p or nonimmune IgG. Chambers were incubated with S3 supernates from either wild-type (WT) or *myo2-66* cells. Only chambers containing anti-Myo2p and wild-type S3 exhibited movement, although the myo2-66p was present in the chamber. No Myo2p was present in the IgG control chamber. (B) Summary of motility results using anti-GFP to adsorb Myo4-GFPp. Immunoadsorbtions were performed from both tagged and untagged strains. Movement was observed only in chambers containing anti-GFP and S3 supernate from Myo4-GFPp–expressing cells.

nonimmune rabbit IgG was used for immunoadsorbtion. Additionally, no movement was observed in chambers containing immunoadsorbed Myo2-66p (Fig 2 A). The presence of Myo2p and Myo2-66p in these chambers (and absence in IgG controls) was confirmed by immunoblot analysis. (Fig. 2 A).

Chicken M5a exhibits optimal motility in the presence of exogenous calmodulin (CaM) and is inhibited in the presence of $Ca^{2+}$ (Reck-Peterson et al., 2000). $Ca^{2+}$ is required (presumably through the action of its CaM light chains) for actin activation of the $Mg^{2+}$-ATPase activity of chicken M5a. Paradoxically, the in vitro motility of this motor is optimal in the absence of $Ca^{2+}$. This is probably due to artifactual effects of CaM light chain loss from the neck domain, since addition of exogenous CaM to motility chambers in both the absence (improves robustness and slightly increases velocity) and presence (prevents inactivation of the motor) of $Ca^{2+}$ augments motility (Reck-Peterson et al., 2000). In contrast, neither addition of exogenous CaM (20 μg/ml) nor 10 μM $Ca^{2+}$(using a 5 mM $Ca^{2+}$/EGTA buffer; Nascimento et al., 1996) had an effect on Myo2p motility (Table II). The lack of $Ca^{2+}$ regulation of Myo2p is not surprising given the phenotypes of yeast CaM or Myo2p neck domain mutants. Yeast CaM mutants do not have a myo2-like phenotype, and myo2 mutants lacking the CaM binding neck domain do not exhibit a myo2 phenotype (Davis, 1992; Stevens and Davis, 1998).

To study Myo4p motility, we used a Myo4p-GFP strain. This is a diploid strain in which both endogenous copies of Myo4p are tagged at the COOH terminus with GFP (Karpova et al., 2000). Myo4p was then immunoadsorbed into motility chambers using an anti-GFP antibody. Myo4p moved actin filaments in vitro at maximum speeds of 1.1 μm/sec. No motility was observed when Myo4p was immunoadsorbed with rabbit nonimmune IgG or when GFP antibodies were used to immunoadsorb from an untagged yeast strain (Fig 2 B). Thus, both Myo2p and Myo4p are active motors.

### *Assays Used to Determine Processivity*

To investigate whether the yeast class V myosins, like chicken M5a, are processive motors, two methods for quantifying the in vitro motility assays were used. The first examines actin filament velocity as a function of motor density. Such assays indicate whether a motor is kinetically equipped to move processively by enabling determination of the duty ratio. Duty Ratio ($f$) is the fraction of the total cycle time ($t_{cycle}$) that a motor remains strongly bound to its track ($t_{on}$): $\sim t_{on}/t_{cycle}$. In these assays, processive motors maintain a constant velocity independent of motor density (Howard et al., 1989), indicating a high duty ratio, whereas the velocity of nonprocessive motors decreases as a function of motor concentration, suggesting a low duty ratio (Uyeda et al., 1990; Harris and Warshaw, 1993). The second assay examines actin filament landing rate as a function of motor density. Landing rate is defined as the number of actin filaments that bind to the motility chamber surface and move for a given field size and time. Such measurements define the stoichiometry of the reaction between motor molecules and the filaments they move along (Howard et al., 1989). For example, for the processive microtubule motor kinesin, or chicken M5a, the relationship between landing rate and motor density demonstrates first

*Table II. Effect of $Ca^{2+}$ and Calmodulin on Myo2p Velocity*

| Condition | Velocity |
|---|---|
| | *μm/s ± SD* |
| EGTA | 2.4 ± 0.45 |
| $Ca^{2+}$ | 2.8 ± 0.36 |
| EGTA/ CaM | 2.7 ± 0.32 |
| $Ca^{2+}$/ CaM | 2.9 ± 0.35 |

power dependence, implying that only one motor is necessary to move a given filament (Howard et al., 1989; Mehta et al., 1999). Nonprocessive motors require more than one motor to move a filament; e.g., five molecules of nonprocessive monomeric kinesin are needed to move a microtubule (Hancock and Howard, 1998).

### *Chick Brain M5a Is a Processive Motor in the Antibody Capture In Vitro Motility Assay*

To verify that the antibody capture assay was a reliable method for studying velocity and landing rate as a function of motor density, we analyzed chicken M5a using this method. Yeast extracts were spiked with 4 μg/ml (the concentration of Myo2p in a soluble yeast lysate) purified chick brain M5a. Chick brain M5a was then immunoadsorbed into motility chambers using antibodies to the tail domain of chick M5a. Chick M5a moved at maximum velocities of 0.8 μm/sec. This is substantially faster than the velocities measured previously (0.3–0.4 μm/sec; Cheney et al., 1993a) for M5a directly adsorbed into motility chambers and may reflect improved steric access resulting from the antibody tether. Alternatively, some factor in the yeast extract may have an activating effect.

To investigate velocity as a function of motor density, velocity was assessed at decreasing amounts of chicken M5a antibody concentrations. The velocity of chick M5a–driven actin filament movement remained constant as a function of antibody concentration even at the lowest concentrations where movements could still be observed (Fig. 3 A). The landing rate was also analyzed as a function of motor density. When landing rate was plotted as a function of antibody concentration, the data were well fit by a function describing a processive motor (see Materials and Methods; Fig. 3 B). Thus, chick M5a behaves as a processive motor using the antibody capture motility assay, recapitulating the results obtained using purified protein adsorbed directly to motility chamber surfaces (Mehta et al., 1999). These results validate the antibody capture motility assay as a means to access the biophysical properties of myosin motors. The chick M5a control was important because, unlike surface adsorption, antibody adsorption of myosin molecules could potentially lead to several motors per site due to the bivalency of antibodies for their ligands and the multivalency of protein A for antibodies. If this had occurred, the landing rate data for M5a would have been best fit with $n < 1.0$. We can rule out this potential artifact because the landing rate data for M5a was best fit with $n = 1.0$.

### *Myo2p and Myo4p Are Processive Actin-based Motors*

Myo2p-driven actin filament velocity and landing rates were examined as a function of Myo2p concentration to determine whether Myo2p is a processive class V myosin. Both velocity and landing rate were measured at varying concentrations of Myo2p tail antibody. To determine the actual concentration of Myo2p at each antibody concentration, quantitative Western blots were performed using Myo2p tail–maltose binding protein fusion protein as a standard (Reck-Peterson et al., 1999). Importantly, there was a linear relationship between the amount of Myo2p immunoadsorbed and the antibody concentration (data not shown). Unlike chick M5a, Myo2p-driven actin filament velocity decreased as a function of Myo2p concentration (Fig. 4 A).

This decrease in motor velocity as a function of Myo2p concentration suggests that Myo2p is a nonprocessive motor. To quantify the processivity of Myo2p, we determined its duty ratio. Processive motors, such as conventional kinesin, have duty ratios of close to 1.0 (Howard et al., 1989), whereas nonprocessive motors have very low duty ratios; for example skeletal muscle myosin II has a duty ratio of 0.04 (Uyeda et al., 1990; Harris and Warshaw, 1993). To determine the Myo2p duty ratio, the average filament velocities were plotted over a range of motor density and

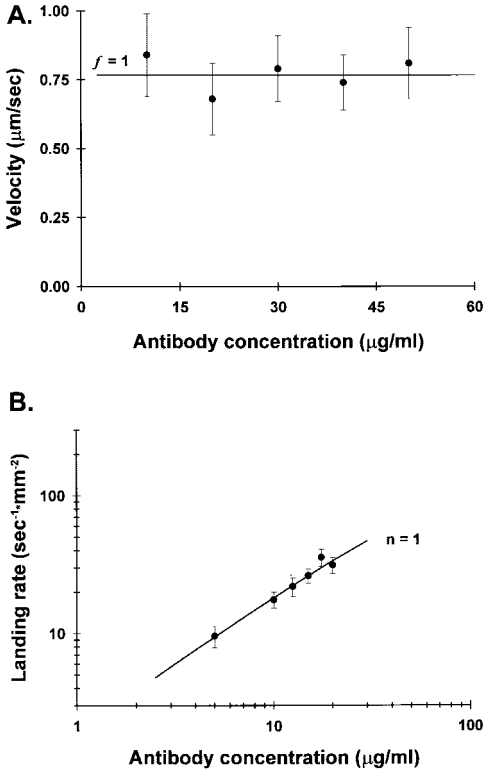


*Figure 3.* Chick brain M5a is a processive motor when assayed using the antibody capture (from S3) in vitro motility assay. (A) Velocity (μm/s ± SD) as a function of M5a tail antibody concentration. No movement was observed at an antibody concentration of 5 μg/ ml. The solid line is a theoretical curve derived from Eq. 1 with the duty ratio, $f = 1$ (see Materials and Methods). The good fit to this line indicates that a single M5a molecule remains strongly bound to an actin filament for nearly its entire catalytic cycle. (B) Landing rate ($n$ ± standard error, where $n$ is the number of landing events per unit area, per unit time; see Materials and Methods) as a function of antibody concentration. The curve fit, derived from Eq. 2, represents the case where $n = 1$, i.e., only one motor molecule is required to bind a filament and initiate movement.

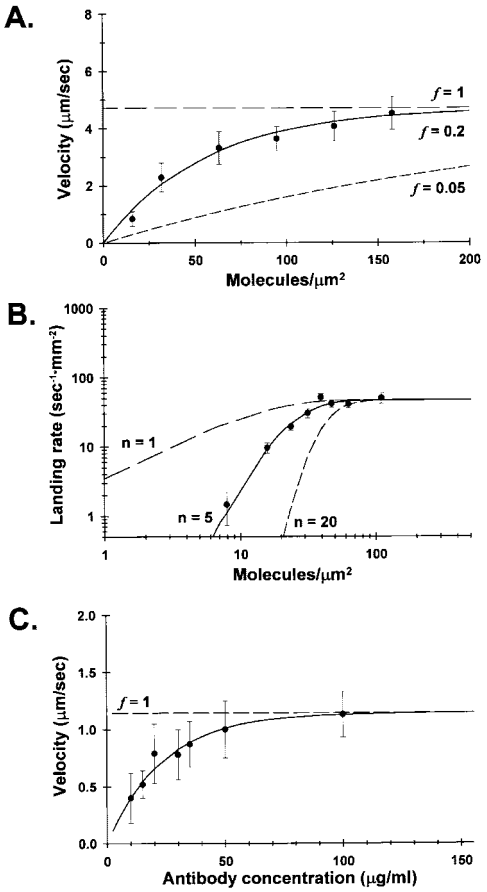


*Figure 4.* Myo2p and Myo4p are nonprocessive class V myosins. (A) Average velocity (μm/s ± SD) is plotted as a function of motor concentration. No movement was seen at a Myo2p concentration of 8.0 molecules/μm$^2$. Myo2p motility data were best fit by the theoretical curve representing a duty cycle, $f = 0.2$ (solid line), indicating that a Myo2p molecule is only strongly bound to actin for ~ 20% of its entire catalytic cycle. Also shown are theoretical curves representing duty ratios of 1.0 (similar to chick brain M5a, long dash) and 0.05 (similar to myosin-II, short dash). (B) Landing rate ($n$ ± standard error, where $n$ is the number of landing events per unit area, per unit time) is plotted as a function of motor concentration. The landing rate was best fit with Eq.4 when $n = 5$ (solid line; see Materials and Methods), indicating that at least five Myo2p molecules are needed to bind and initiate the movement of an actin filament. Also shown are theoretical landing rate fits where $n$ is equal to 1 (a processive motor such as M5a, long dash) or 20 (a highly nonprocessive motor, short dash). (C) Myo4p velocity (μm/s ± SD) as a function of anti-GFP antibody concentration. No movement was observed using an antibody concentration of 5 μg/ml.

fit to Eq. 1. The results indicate that the maximum duty ratio for Myo2p is ~0.2, a value significantly lower than that expected for a processive motor, but still significantly higher than values observed for the highly nonprocessive motor, myosin-II (duty ratio = 0.04; Fig. 4 A).

The landing rate data for Myo2p was fit to Eq. 2, a model originally developed by Hancock and Howard (1998) to examine the landing rate of kinesin (see Materials and Methods). In this model, $n$ represents the number of molecules needed to bind a filament and produce motion. The Myo2p landing rate data was best fit to this model when $n = 5$, indicating that at least five Myo2p molecules are necessary to produce filament movement (Fig. 4 B). Interestingly, this value is similar to that predicted to be the theoretical lower limit for the number of Myo2p molecules needed to achieve maximum velocity (~1/duty ratio = 5). Thus, Myo2p appears to be an intermediate duty ratio, nonprocessive class V motor.

To investigate the motor properties of Myo4p, velocity as a function of antibody concentration was examined. Like Myo2p, the velocity of Myo4p-driven movement decreased as antibody concentration decreased, suggesting that Myo4p, like Myo2p, is also a nonprocessive motor (Fig. 4 C). Because we do not have a Myo4p antibody and our GFP antibodies did not detect Myo4p by immunoblot, we were unable to obtain the duty ratio and landing rate for Myo4p. Thus, we can conclude qualitatively, but not quantitatively that Myo4p is also a nonprocessive class V myosin.

The finding that Myo2p and Myo4p have very different motor properties when compared with chick brain M5a is novel because this is the most dramatic example of myosins within the same class having different duty ratios. For example, both smooth and skeletal muscle myosin-II have very low duty ratios (~0.04) (Uyeda et al., 1990; Harris and Warshaw, 1993), and myosins-I have been reported to have duty ratios that vary from 0.04 (Ostap and Pollard, 1996) to 0.15 (Jontes et al., 1997; Coluccio and Geeves, 1999). Sequence similarity within the motor domains was used to originally establish the different myosin classes (Cheney et al., 1993b). One expectation of class assignment was that the motor properties within a class would be similar. Here, we have shown that this is not the case; motor properties of individual myosins may differ dramatically within classes. The in vitro motility assay and data analysis used in this paper should be useful for the characterization of novel myosin proteins and myosins that cannot or have not yet been purified to homogeneity.

The finding that both Myo2p and Myo4p are low duty ratio motors has implications for their proposed biological functions. To directly transport membranes, as has been proposed for Myo2p or an RNP and for Myo4p (Reck-Peterson et al., 2000), several molecules (at least five for Myo2p) must be present per cargo molecule. It is also possible that the yeast class V myosins are processive motors in vivo due to a regulatory event. For instance, binding of the dynactin complex to the microtubule motor dynein increases dynein's processivity (King and Schroer, 2000). A candidate protein for regulating the motor properties of Myo2p is the small GTPase, Rho3p, which has been shown to bind to the neck domain of Myo2p (Robinson et al., 1999). Preliminary experiments using recombinant Rho3p suggest that Rho3p does not regulate the motor activity of Myo2p (S. Reck-Peterson, unpublished results). Given the large number of genetic interactions between the Rab protein, Sec4p, and Myo2p, it is also possible that Sec4p could modulate the processivity of Myo2p.

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