She3p Binds to the Rod of Yeast Myosin V and Prevents It from Dimerizing, Forming a Single-headed Motor Complex*

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Vertebrate myosin Va is a dimeric processive motor that walks on actin filaments to deliver cargo. In contrast, the two class V myosins in budding yeast, Myo2p and Myo4p, are non-processive (Reck-Peterson, S. L., Tyska, M. J., Novick, P. J., and Mooseker, M. S. (2001) J. Cell Biol. 153, 1121–1126). We previously showed that a chimera with the motor domain of Myo4p on the backbone of vertebrate myosin Va was processive, demonstrating that the Myo4p motor domain has a high duty ratio. Here we examine the properties of a chimera containing the rod and globular tail of Myo4p joined to the motor domain and neck of mouse myosin Va. Surprisingly, the adaptor protein She3p binds to the rod region of Myo4p and forms a homogenous single-headed myosin-She3p complex, based on sedimentation equilibrium and velocity data. We propose that She3p forms a hetero coils-coil with Myo4p and is a subunit of the motor. She3p does not affect the maximal actin-activated ATPase in solution or the velocity of movement in an ensemble in vitro motility assay. At the single molecule level, the monomeric myosin-She3p complex showed no processivity. When this construct was dimerized with a leucine zipper, short processive runs were obtained. Robust continuous movement was observed when multiple monomeric myosin-She3p motors were bound to a quantum dot “cargo.” We propose that continuous transport of mRNA by Myo4p-She3p in yeast is accomplished either by multiple high duty cycle monomers or by molecules that may be dimerized by She2p, the homodimeric downstream binding partner of She3p.

It is well established that vertebrate myosin Va is a highly processive motor protein that can move for several μm without dissociating from its actin track (reviewed in Ref. 1). Efficient processivity requires several features, including a high duty cycle motor domain (2, 3) and strain-dependent kinetics that put the two heads kinetically out of phase to ensure continuous forward stepping (4–6). The wealth of data on vertebrate myosin Va led one to believe that processivity was a property common to all class V myosins. This assumption was challenged with the observation that neither of the two class V myosins from the budding yeast Saccharomyces cerevisiae, perversely called Myo2p and Myo4p, are processive motors (7). Drosophila myosin V (8) and, more recently, human myosin Vc (9) have also been reported to be nonprocessive.

Is a processive motor necessary to fulfill the biological role of yeast myosin V? Myo2p has a number of cargoes, including vacuoles, late Golgi, peroxisomes, and secretory vesicles, the last of which is its essential cargo (reviewed in Ref. 10). Myo4p transports over 20 different mRNAs (11, 12) and cortical endoplasmic reticulum (13, 14) to the bud on actin cables. One of the best studied cargoes of Myo4p is ASH1 mRNA, which preferentially accumulates in the daughter cell and prevents it from switching mating type (15, 16). Three of the proteins required for proper localization of ASH1 mRNA are Myo4p (also known as She1p), She2p, and She3p. She3p acts as an adaptor between the motor and She2p, which binds the mRNA cargo (reviewed in Ref. 17).

Our goal is to understand the molecular basis for the lack of yeast Myo4p processivity. Myo4p was reported to be nonprocessive, because it had a low to intermediate duty ratio (7), whereas a processive dimer requires that each head have a duty ratio of >50%. We engineered a chimera that contained the Myo4p motor domain on the neck and rod of mouse myosin Va, with the expectation that it would be nonprocessive. Surprisingly, this chimera showed even longer processive run lengths than wild type mouse myosin Va at low ionic strength (18). This result established that the Myo4p motor domain has a high duty cycle and the potential to support processive motion. Based on this result, we proposed that the poor α-helical coiled-coil propensity of the Myo4p rod allows it to exist as a nonprocessive monomer (18). The propensity to form α-helical coiled-coil is based on the strength of the heptad repeat, a seven-amino acid motif in which the first and fourth positions, which form the internal seam of the coiled-coil, are hydrophobic amino acids. The rod of dimeric mouse myosin Va has a total of ~60 heptad repeats of coiled-coil, and the first ~20 heptad repeats before the PEST site are sufficient to ensure dimerization (Fig. 1a). In contrast, the Myo4p rod has a total of about five heptads of potential α-helical coiled-coil, which may be insufficient to support dimerization (Fig. 1a) (19). Here we test this hypothesis by analyzing the properties of a chimera containing the motor domain and neck of mouse myosin Va, a known processive motor, joined to the rod and globular tail of Myo4p.

The results show that the Myo4p rod has little tendency to associate with itself and form a dimer, an observation not made with any other class V myosin to date. The coiled-coil region of

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the Myo4p rod instead prefers to interact with its binding partner, She3p, probably forming a heterocoiled-coil. The myosin-She3p complex forms a homogeneous single-headed motor. She3p is thus not a typical binding partner but an intrinsic part of the Myo4p molecule. Processive runs were only observed if the myosin-She3p complex was dimerized with a leucine zipper, creating a two-headed motor, or when multiple motors were bound to the Qdot “cargo.” In the yeast cell, mRNA transport may be driven by multiple monomeric motors. Alternatively, we speculate that Myo4p may be dimerized to form a processive motor by She3p, the homodimeric downstream binding partner of She3p (20).

**EXPERIMENTAL PROCEDURES**

**Myosin V Constructs**—Four chimeras were engineered, each of which contains the motor domain and neck of mouse myosin Va (amino acids Met1–Lys908; accession number NP034994). This head was joined to four variations of the rod and globular tail of Myo4p from *S. cerevisiae* (Fig. 1b). All constructs, including the mouse myosin V HMM control, contained a C-terminal FLAG tag to facilitate purification and a biotin tag at the N terminus for attachment to streptavidin quantum dots (Qdots) (Invitrogen), as described in Ref. 21. The full-length constructs contained amino acids Glu928–Gln1026 of Myo4p. The joining sequence between the end of mouse myosin V and the beginning of the Myo4p rod is RELKKLKERNNIRAS, with Myo4p amino acid sequence shown in italic type. For the full-length-Zip and ΔGT-Zip chimeras, a leucine zipper was added after the predicted coiled-coil region of the Myo4p rod, following amino acid Ile1025 to Lys1471, and then by a FLAG-tag. The plasmid vector pAcSG2. The *S. cerevisiae* genome was amplified by PCR from Beningo) was used as the PCR template for these constructs. SHE3 with a C-terminal His tag was cloned into the baculovirus vector pAcSG2. The SHE3 gene was amplified by PCR from *S. cerevisiae* genomic DNA and verified to be the coding sequence found at the Saccharomyces genome data base.

**Protein Expression and Purification**—Myosin constructs were expressed in S9 cells using the baculovirus system and purified on a FLAG affinity column as described previously (18). The myosin constructs were co-expressed with calmodulinΔall (CaMΔall), a mutant lacking all calcium binding sites (22). For one experiment that tested the effect of calcium binding on activity, the heavy chain was co-expressed with wild-type calmodulin. CaMΔall was also expressed in bacteria (22). In order to ensure that all IQ motifs were occupied, 3.0 μM excess CaMΔall was added to the cell lysate during purification, and 6.0 μM CaMΔall was added to the final buffer for all assays except analytical ultracentrifugation. Where indicated, virus encoding for She3p was also included in the co-infection. Chicken skeletal actin was prepared from acetone powder (23).

**Analytical Ultracentrifuge**—An Optima XL-I analytical ultracentrifuge (Beckman Coulter) was used to determine the sedimentation coefficients of the expressed constructs. Sedimentation velocity runs were performed in the An60Ti rotor at 35,000 rpm and 20 °C, in 10 mm Hepes, pH 7.0, 0.2 m NaCl, 1 mM DTT, 1 mM EGTA, 1 mM NaN3, and ~2 μM myosin. Sedimentation values were corrected for density and viscosity of the solvent. Sedimentation coefficients were determined by curve fitting to one or more species, using the dc/dt program (24). Sedimentation equilibrium data were collected at 6,000 rpm and 4 °C, 10 mM Hepes, pH 7.0, 0.2 m NaCl, 1 mM DTT, 1 mM EGTA, 1 mM NaN3, and ~1.5–4 μM myosin. The data were analyzed with Origin software provided with the Beckman Optima XL-I ultracentrifuge. For molecular weight calculations, the fit to the average of three independent scans at equilibrium was determined for each protein sample, and the average and S.D. of those values was calculated for each construct.

**Steady-state Actin-activated ATPase Assay**—NADH-linked actin-activated ATPase assays were performed at 30 °C, 10 mM imidazole, pH 7.4, 50 mM KCl, 1 mM MgATP, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 1 mM NaN3, 50–100 nM myosin, and 6.0 μM CaMΔall as previously described (18).

**In Vitro Motility**—The myosin V constructs (0.1 mg/ml) were mixed with a 2-fold molar excess of F-actin and 1 mM MgATP in a high salt buffer (25 mM imidazole, pH 7.4, 0.3 M KCl, 4 mM MgCl2, 1 mM EGTA, 10 mM DTT) and centrifuged for 20 min at 400,000 × g to remove myosin that was unable to dissociate from actin in the presence of ATP. The concentration after the spin was measured by the Bradford Protein Assay. The myosin was then diluted into the same buffer used for centrifugation and adsorbed directly to nitrocellulose-coated coverslips that were preincubated with 0.01 mg/ml bovine serum albumin for 30 s. Rhodamine-phalloidin labeled actin filaments were passed through a 21½ gauge needle to fragment them to ~1 μm in length. The motility assay was performed at 30 °C in Motility Buffer (25 mM imidazole, pH 7.4, 50 mM KCl, 1 mM MgATP, 4 mM MgCl2, 1 mM EGTA, 50 mM DTT), that also contained 6.0 μM CaMΔall, an oxygen-scavenging system (3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.18 mg/ml catalase), and an ATP-regenerating system (0.5 mM phosphoenolpyruvate and 100 units/ml pyruvate kinase).

**Total Internal Reflection Fluorescence (TIRF) Microscopy**—Single molecule motility assays using TIRF microscopy were performed as described previously (21, 25). For single molecule experiments, the myosin V constructs were diluted to 0.2 μM (concentration of heads) and mixed with a 10-fold molar excess of 655-nm streptavidin-coated quantum dots (Invitrogen) to ensure single motor transport. Because the constructs were labeled with a biotin tag at the N terminus of the motor domain, it is unlikely that multiple motors could simultaneously transport a single quantum dot. For multiple motor experiments, the full-length chimeras were diluted to 0.2 μM and mixed with 655-nm carboxylated quantum dots (Invitrogen) at a ratio of 4 or 8 heads per quantum dot. Rhodamine-phalloidin labeled actin was attached to a glass flow cell using N-ethylmaleimide-modified skeletal muscle myosin, which forms a strong and ATP-insensitive bond with actin. The Qdot-labeled myosin...
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FIGURE 1. Probability of coiled-coil formation for Myo4p compared with mouse myosin Va and schematic representation of the four chimeras. a, α-helical coiled-coil probability for the Myo4p rod (amino acids 928–1062) and the mouse myosin Va rod (amino acids 904–1474). Paircoil analysis predicts only 5 heptads of potential coiled-coil in the rod of Myo4p, with a maximum probability of 60%, whereas there are ~60 heptads of potential coiled-coil in the mouse rod, with a maximum predicted probability of 100%. b, in all chimeras, the motor domain and IQ motifs are from mouse myosin Va, shown in white. For the full-length chimeras, the rod (light gray) and the globular tail (GT, dark gray) are from Myo4p. The ΔGT chimeras are similar to full-length but lack the globular tail. A version of both the full-length and ΔGT chimeras was made with a leucine zipper (black) following the rod domain, ensuring constitutive dimers.

FIGURE 2. She3p co-purifies with the full-length and ΔGT chimeras. SDS-PAGE of the full-length, ΔGT, and mouse myosin V constructs after purification on a FLAG affinity column. Each construct was co-expressed with or without She3p to test for binding, and all constructs were co-expressed with calmodulin. Lane 1, molecular weight markers; lanes 2 and 3, the full-length chimera co-expressed with She3p or in its absence. Lanes 4 and 5, the ΔGT chimera co-expressed with She3p or in its absence. Lane 6, mouse myosin Va HMM co-expressed with She3p. She3p co-purifies with the full-length and ΔGT chimeras in a 1:1 molar ratio with the heavy chain but does not co-purify with mouse myosin V.

was then diluted to between 0.25 and 2 nm in Motility Buffer (see above) with 1 mg/ml bovine serum albumin, 6.0 μM CaM2aI, an oxygen-scavenging system, and an ATP-regenerating system and added to the flow cell. Through-the-objective TIRF microscopy was performed at 30 °C. The final pixel resolution was 55 nm, and data were collected at 4–12 frames/s.

Myosin movement along fluorescently labeled actin filaments was tracked by hand, using the program ImageJ. For each event, we required the quantum dot-labeled construct to move continuously for at least 4 frames in order to qualify as a run. The characteristic run length λ was determined by nonlinear least squares fitting of the equation $P(x) = A e^{-x/\lambda}$ to the run length distribution, where $P(x)$ represents the probability of the myosin traveling a distance $x$ along an actin filament, and $A$ is a constant.

RESULTS

Expressed Constructs—Two chimeric constructs were expressed in the baculovirus/Sf9 cell system to test if the rod region of Myo4p can dimerize with itself by forming a coiled-coil (Fig. 1b). The first, called full-length chimera, contained the rod and globular tail (GT) of yeast Myo4p, joined to the motor domain and neck of mouse myosin Va. The second, called the ΔGT chimera, lacked the globular tail and contained only the rod of Myo4p. An additional version of each construct was engineered by the addition of a leucine zipper after the Myo4p rod, thus ensuring that these two constructs are dimeric, two-headed motors. The leucine zipper is an ~30-amino acid sequence that forms a particularly stable α-helical coiled-coil, primarily due to the presence of Leu residues at the fourth position of each heptad repeat. SDS-gels of the purified proteins are shown in Fig. 2. All of the chimeric constructs expressed well in the baculovirus/insect cell expression system, whereas intact native Myo4p could only be expressed in low yield, precluding its detailed characterization at present. Because all IQ motifs are not equal, we chose to retain the mouse IQ motifs in the chimeras. This allowed mammalian CaM to abut the motor domain instead of yeast CaM, which is only 58% identical to the mammalian isoform. The Myo4p IQ motifs bind only yeast CaM, and we did not want to perturb the known processive nature of the myosin Va motor. This strategy allows us to assess only the effect of the Myo4p rod on a known processive motor.

Dimerization Properties of the Myo4p Rod—The full-length chimera showed a marked tendency to aggregate when analyzed by sedimentation velocity (Fig. 3a). Three-fourths of the total protein formed large aggregates that rapidly sedimented; the remainder of the protein could be fit to a homogeneous species that sedimented at ~7 S (Table 1). Sedimentation equilibrium experiments were performed on protein from which the aggregates were removed, either by gel filtration or by clarification at high speed. The values were more similar to a monomer than a dimer (Table 1), showing that this construct forms a range of species from monomers to higher oligomers.

The aggregation shown by the full-length chimera was essentially abolished in the ΔGT chimera (Fig. 3c). The molecular weight of this species, determined by sedimentation equilibrium, was that of a dimer (Table 1). The dimer sedimented at 8.8 S, consistent with a higher molecular weight and increased asymmetry compared with the ~7 S full-length monomer. In the absence of the globular tail, the coiled-coil region of the rod can therefore dimerize with itself at protein concentrations greater than 1 μM. Only the construct lacking the globular tail formed a homogeneous dimer, implying that the globular tail may have the potential to interact with the coiled-coil-forming...
region, inhibiting dimerization by a mechanism similar to that suggested for myosin VI (26).

She3p Stabilizes a Single-headed Motor Complex—We co-expressed the FLAG-tagged full-length and ΔGT chimeras with HIS-tagged She3p. She3p co-purified with both constructs when isolated from Sf9 cell lysates on a FLAG affinity column (Fig. 2). By gel densitometry, 0.98 ± 0.04 mol of She3p bound per mol of heavy chain of the ΔGT construct (supplemental Fig. S1). These results localize the primary binding site of She3p to the rod region of Myo4p and establish that the binding of She3p to the Myo4p is tight, as indicated by its co-purification from a dilute (∼0.2 μM) lysate. It was recently shown that Myo4p purified from yeast also has She3p bound, in agreement with our observations (27). She3p did not co-purify with mouse myosin V HMM (Fig. 2, lane 6), which rules out the possibility that She3p binds to the head or neck of the chimeras.

The presence of She3p caused a striking change in the sedimentation velocity profile of the full-length chimera (Fig. 3b). The large aggregates were essentially abolished, and the predominant species sedimented at the same value as the minor species in the absence of She3p (Table 1). Importantly, the molecular weights obtained with the full-length-She3p complex were consistent with a species consisting of one molecule of each of the two proteins, to create a homogeneous, heterodimeric single-headed motor (Fig. 4a and Table 1). This result implies that the Myo4p rod prefers to interact with a molecule of She3p rather than a second Myo4p rod.

With the ΔGT chimera, She3p again caused the formation of a single-headed motor species in complex with one molecule of She3p, as determined from sedimentation equilibrium data (Fig. 4b and Table 1). She3p had little effect on aggregation of the ΔGT chimera, which in its absence was already fairly homogeneous. The ΔGT-She3p complex sedimented at 6.4 S (Table 1), compared with 8.8 S in the absence of She3p, consistent with its lower molecular weight as a complex of one heavy chain and one molecule of She3p, versus two heavy chains. Because She3p abolished the dimerization seen with ΔGT alone, the Myo4p rod has a higher affinity for She3p than it does for a second Myo4p rod. This is the first example of a heterologous protein causing a myosin motor to exist as a single-headed species.

She3p Does Not Affect Actin-acti-
vated ATPase Activity or Ensemble

![Graphs](image)

**FIGURE 3.** Sedimentation velocity of the full-length and ΔGT chimeras. a–c, absorbance as a function of distance and time for the full-length chimera (a), the full-length-She3p complex (b), and the ΔGT chimera (c). Each trace represents the absorbance profile at one point in time. Multiple traces at subsequent intervals are overlaid. The rapid decrease and heterogeneous shape of the absorbance profiles for the full-length construct shows that multiple species of oligomers are formed, which sediment rapidly. In contrast, the full-length-She3p and ΔGT constructs were predominantly fitted by a single species. d, sedimentation coefficient of the full-length-She3p complex determined by curve-fitting to one species, using the dc/dt program (24). The quality of the fit indicates that a homogeneous species is present. Sedimentation coefficients of these and other constructs are summarized in Table 1.

**TABLE 1**
Molecular weights and sedimentation coefficients

| Construct          | She3p<sup>a</sup> | Calculated molecular mass<sup>b</sup> | Observed molecular mass | S<sub>n/70</sub><sup>c</sup> |
|--------------------|-------------------|--------------------------------------|-------------------------|---------------------------|
|                    |                   | Monomer Dimer                        |                         |                           |
|                    |                   | kDa                                  | dS                               |
| Full-length        | –                 | 277                                  | 372 ± 52 (n = 5); 335 ± 42 (n = 3)<sup>d</sup> | 7.2 ± 0.4 (n = 5)         |
|                    | +                 | 325                                  | 321 ± 31 (n = 6)               | 7.5 ± 0.6 (n = 4)         |
| ΔGT                | –                 | 226                                  | 432 ± 44 (n = 6)               | 8.8 ± 0.2 (n = 3)         |
|                    | +                 | 275                                  | 317 ± 27 (n = 4)               | 6.4 (n = 1)               |
| ΔGT-Zip            | –                 | 460                                  | 472 ± 35 (n = 3)               | 8.7 ± 0.2 (n = 3)         |
|                    | +                 | 557                                  | 507 ± 20 (n = 3)               |                           |
| Full-length-Zip<sup>d</sup> | – | 562                                  | 457 ± 51 (n = 6)               | 8.1 (n = 1)               |

<sup>a</sup> Indicates whether or not She3p was co-expressed with the heavy chain construct.

<sup>b</sup> Monomer weight is calculated for one heavy chain including tags, six calmodulins, and one She3p when present.

<sup>c</sup> Sedimentation coefficient of the smallest species.

<sup>d</sup> Protein analyzed following gel filtration.
Motility—The actin-activated ATPase activity of the two constructs was determined in the presence or absence of She3p. Despite the ability of She3p to form a single-headed motor complex, it had very little effect on the activity of either the full-length or the ΔGT chimera (Fig. 5a and Table 2). The maximal activity of the ΔGT chimera was ~3 times greater than that of the full-length chimera, independent of She3p. An enhancement of activity with a truncated construct generally suggests that a regulatory element has been deleted. In mouse myosin Va, the addition of calcium opens the molecule from its compact, inhibited form to its unfolded, active form, increasing the ATPase rate (22). However, the addition of calcium did not increase the activity of the full-length chimera expressed with wild-type calmodulin (data not shown). Nonetheless, the full-length chimera does not express the full activity that the mouse motor domain is capable of, implying that some inhibitory interaction involves the globular tail.

We also measured the speed at which the full-length (Fig. 5b) and ΔGT chimeras (supplemental Fig. S2) move actin filaments in the in vitro motility assay, as a function of motor concentration. The motility is nearly identical for the full-length and ΔGT chimeras, suggesting that when the full-length chimera is bound to a surface, the inhibition seen in solution is abolished. For both constructs, the motility is also unaffected by the presence of She3p, consistent with the ATPase results. Adding a leucine zipper reduced the speed significantly. The speed of the chimeras decreases as motor concentration increases, by almost 50% at high motor concentrations. This concentration dependence is very unusual (see “Discussion”). In contrast, the speed of mouse myosin Va and other processive motors is independent of motor concentration (Fig. 5b, open squares) (28, 29).

The fact that the full-length chimera had the same motility as ΔGT, but lower ATPase activity, implies that binding the full-length construct to the surface of the motility chamber may activate the molecule, as it does for mouse myosin Va (22). She3p Abolishes Processivity, but Heavy Chain Dimerization Restores It—Because each construct contains the motor domain and neck of the highly processive mouse myosin Va, we expect to see processive movement if the Myo4p rod is capable of dimerizing. The motor domains of single full-length or ΔGT molecules were labeled with streptavidin-coated quantum dots via a biotin tag at the N terminus of the motor domain. TIRF microscopy was then used to track the molecules as they walked along fluorescently labeled actin filaments attached to a coverslip. For each construct, the characteristic run length λ, the average speed, and the total number of processive runs per data file were determined (Table 3).

The ΔGT chimera was processive, but the runs were shorter, slower, and much less frequent than those of mouse myosin V.
The low frequency of processive runs suggests that at the nanomolar concentrations used in the processivity assay, the ΔGT chimera is predominantly monomeric with a small population of dimers, whereas at the micromolar concentrations used for molecular weight determinations, the construct is primarily of dimers, whereas at the micromolar concentrations used for molecular weight determinations, the construct is primarily monomeric with a small population of dimers. Dimerization of the entire population with a leucine zipper clearly increases the frequency of processive runs and overrides the ability of She3p to create a nonprocessive monomer. The speed of all of the chimeras was slower than mouse myosin Va, implying that the rod can affect kinetics. This might be a result of the short rod of Myo4p. A mouse myosin Va HMM truncated after five heptads of coiled-coil and dimerized by a leucine zipper also moved processively along actin at approximately half the speed of standard (~20 heptads coiled-coil) mouse HMM (data not shown).

Similar results were obtained for the full-length chimera, but the run lengths and frequency of runs were lower than for ΔGT, making data analysis difficult (Table 3). Occasional processive runs were observed for the full-length chimera, probably due to oligomers, but never with the monomeric full-length-She3p complex. The dimerized full-length-Zip chimera was also weakly processive and insensitive to the presence of She3p. The reduced run lengths and frequencies of the full-length chimera suggest that the globular tail plays an inhibitory role.

**TABLE 2**

| Construct       | She3p* | \( V_{max} \) \( \mu M \) | \( K_m \) \( \mu M \) | \( n \) |
|-----------------|--------|-----------------------------|-------------------------|-------|
| Full-length     | -      | 6.4 ± 0.7                   | 10.3 ± 3.9              | 3     |
| ΔGT             | +      | 4.7 ± 1.5                   | 7.5 ± 2.4               | 4     |
| Full-length-Zip | -      | 11.3 ± 0.8                  | 2.2 ± 0.3               | 5     |
| ΔGT-Zip         | +      | 13.7, 12.8                 | 2.4, 3.3                | 2     |

*a* Indicates whether or not She3p was co-expressed with the heavy chain construct. *b* \( V_{max} \) values are calculated per head, assuming that 1 mol of She3p is bound per mol of heavy chain when it is present.

**TABLE 3**

| Construct       | She3p* | Run length \( \mu m \) | Speed \( \mu m / s \) | Runs/file |
|-----------------|--------|------------------------|----------------------|-----------|
| ΔGT             | -      | 0.49 ± 0.16            | 0.32 ± 0.20          | 1.3       |
| ΔGT-Zip         | -      | 0.46 ± 0.02            | 0.35 ± 0.23          | 18        |
| Full-length     | +      | 0.54 ± 0.01            | 0.38 ± 0.21          | 16        |
| Full-length-Zip | -      | ND                     | 0.37 ± 0.14          | 0.9       |
| Mouse HMM       | +      | ND                     | 0.25 ± 0.10          | 4.4       |

*a* Indicates whether or not She3p was co-expressed with the heavy chain construct.

**TABLE 4**

| Construct       | She3p* | Run length \( \mu m \) | Speed \( \mu m / s \) | \( n \) |
|-----------------|--------|------------------------|----------------------|-------|
| Full-length     | -      | 0.91 ± 0.13            | 0.56 ± 0.27          | 32    |
| Full-length-Zip | +      | 0.84 ± 0.04            | 0.59 ± 0.23          | 134   |

*a* Indicates whether or not She3p was co-expressed with the heavy chain construct.

**Multiple Single-headed Full-length-She3p Motors Support Robust, Continuous Cargo Transport**—To first establish that multiple high duty cycle monomeric motors can continuously move a quantum dot along an actin filament, we engineered a monomeric mouse myosin Va, consisting of the motor domain and six IQ motifs, with a biotin tag at the C terminus. Myosin was attached to streptavidin-coated Qdots at a ratio of 10 motors/Qdot. Long continuous runs of several \( \mu m \) were observed. The data appeared to follow a single exponential distribution and yielded a characteristic run length of 2.5 ± 0.2 \( \mu m \) (supplemental Fig. S3), compared with 1.0 ± 0.2 \( \mu m \) for a single myosin Va dimer.

To test whether multiple monomeric full-length-She3p motors could also support continuous movement, myosin was attached to carboxylated quantum dots at a ratio of 8 motors/Qdot. Quantum dot-motor complexes moved continuously for distances up to several \( \mu m \) before dissociating from actin filaments (Fig. 6, a and b). The full-length chimera without She3p also showed robust, continuous movement (Table 4).
Single-headed Class V Myosin

The run lengths appeared to follow a single exponential distribution and yielded a characteristic run length of 0.84 ± 0.04 μm (Table 4). Note that we did not necessarily expect the run lengths to follow a single exponential distribution, because the number of motors per Qdot and the number of motors able to simultaneously interact with an actin filament varies throughout the population. Nonetheless, this value is longer than the run lengths obtained with single molecules of dimeric ΔGT and almost as long as the run length of single mouse myosin Va dimers. Even longer runs (characteristic run length = 1.7 ± 0.2 μm) were obtained with the full-length-Zip-She3p complex, despite a lower mixing ratio of 4 heads (2 dimers)/Qdot. The run lengths for the full-length constructs were much longer with carboxylated Qdots than with streptavidin Qdots. This is at least partially due to the effect of multiple motors, but it might also indicate the carboxylated Qdot activated the construct by binding to the globular tail, as we have observed for full-length mouse myosin Va. The ΔGT constructs could not be similarly assessed, because a globular tail domain is required to bind to the carboxylated Qdots, based on studies with truncated and full-length constructs of mouse myosin Va. These results clearly demonstrate that multiple monomeric motors are capable of supporting long range continuous transport.

DISCUSSION

Here we show that the rod region of Myo4p does not form an α-helical coiled-coil with itself but preferentially associates with its downstream binding partner She3p, resulting in a single-headed motor complex. She3p is not a typical cargo adaptor protein but an intrinsic part of the Myo4p motor. Although most adaptor molecules bind to the globular tail, the primary binding site of She3p is the rod region. Paircoil analysis of the N-terminal half of She3p, the domain that interacts with Myo4p (30), shows that it has regions of potential coiled-coil, just as the Myo4p rod does. We therefore propose that She3p and Myo4p form a heterocoiled-coil. This would simultaneously account for the strong binding of the two proteins to each other and provide a mechanism by which She3p inhibits dimerization of Myo4p with itself. The light chain of kinesin also interacts with the heavy chain via a coiled-coil motif and is considered to be a subunit of this motor, although it has no effect on the ability of kinesin to dimerize (31). Why is the Myo4p adaptor protein so tightly bound to the motor? Myo4p has only two known cargoes, mRNA and cortical endoplasmic reticulum, and both require She3p (13). Only motors that transport multiple cargoes, such as Myo2p from yeast or vertebrate myosin Va, would require the ability to interchange adaptor proteins in order to carry different cargo at different times. Myo4p has thus been perfectly tuned for its biological role.

Another finding is that the most robust and longest continuous transport along actin filaments was observed when multiple single-headed motors or multiple forced dimers were bound to the quantum dot “cargo.” For long continuous runs to occur, at least one head must remain bound to the cargo at all times. In vertebrate myosin Va dimers, internal strain between the two heads alters the kinetics to keep the front head from dissociating prematurely (4–6). In the case of multiple monomeric motors, there is probably no strain-based coordination between the heads. To increase run lengths then requires increasing the number of motors and/or increasing the duty ratio of the motor (3). Based on the geometry of the Qdot (20-nm diameter), probably only three or four heads on a Qdot can simultaneously interact with a single actin filament. The motor domains of both Myo4p and myosin Va have a high duty ratio (18), and thus several heads should be sufficient for long runs (3).

Short processive runs were observed from single molecules of constructs that were forced to dimerize with a leucine zipper. The run lengths obtained from a single dimer are significantly less than the length of a yeast cell. They are also shorter than the ~1.0-μm run lengths obtained with wild type mouse myosin Va, despite the fact that the two constructs share the same neck and motor domain. This is probably because the chimeric dimers lack the full strain-dependent head-head coordination of vertebrate myosin Va, where the coiled-coil region begins soon after the last IQ motif in the neck. In contrast, the first ~70 amino acids of the Myo4p rod are not predicted to form a coiled-coil, so this region may not transfer strain efficiently between the two heads. This idea is supported by the observation that the dimeric ΔGT chimera has the same ATPase rate per head as the monomeric ΔGT-She3p complex. Mouse myosin Va dimers have a lower ATPase rate per head than monomeric constructs, due to the strain-mediated coordination between the heads (21). As expected, one molecule of the single-headed Myo4p-She3p complex is not processive.

Reduced Speed at High Motor Density—The speed at which the ΔGT and full-length chimeras move actin in an ensemble motility assay decreases as myosin concentration is increased, implying that multiple motors interfere with each other. The speed of mouse myosin Va HMM, in contrast, is independent of myosin concentration. In agreement with our observations, a concurrent study showed that wild-type Myo4p purified from yeast showed a similar concentration dependence in the motility assay, as did a chimeric construct containing the Myo2p motor domain on the Myo4p backbone (27). The Myo4p rod is thus the structural element conferring this behavior. In contrast, the first demonstration of Myo4p in vitro motility showed a decreased actin gliding speed at lower myosin concentrations, which led the authors to suggest that the Myo4p duty ratio was not high enough to support processive movement (7).

One possible explanation for this unusual concentration dependence involves the effects of strain on the ADP release rate, which is increased by forward strain and decreased by backward strain (4, 5). In the motility assay, attached heads sense the external strain imposed by the other heads attached to the same filament. Strain will have a net effect if its effects on each head are asymmetric (4). If backward strain dominates, then more attached motors lead to reduced ADP release rates and thus lower speeds. Consistent with this idea, the in vitro motility of monomeric mouse myosin Va containing six IQ motifs displays a similar concentration profile, with the speed gradually dropping by 50% as myosin concentration was increased (supplemental Fig. S4). A similar argument holds for the dimeric zippered constructs, because they probably lack the strain-dependent head-to-head coordination of dimeric mouse myosin V. The kinetics of the two heads of vertebrate myosin V are more sensitive to the internal strain within the molecule.
of the Myo4p rod is unknown.

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FIGURE 7. Possible mechanisms of mRNA transport. a, if Myo4p is monomeric during mRNA transport in vivo, then multiple motors are necessary. ASH1 mRNA has four zip codes and can thus bind four motors. b, Myo4p might operate as a dimeric, processive motor in vivo. Downstream binding partners, such as She2p, might provide a scaffold for dimerizing Myo4p. In this case, a single zip code might be sufficient for long range transport.

(32), particularly in the motility assay, where external strain is relatively small.

Alternatively, one could invoke a lack of flexibility at the lever arm-rod junction. For multiple motors oriented randomly on a substrate to cooperate in moving a single actin filament, the motors must be flexible. For vertebrate myosin Va, there is a flexible hinge at the junction between the lever arm and the coiled-coil (33), and the motility is independent of motor concentration. Similarly, the motility of conventional kinesin is independent of concentration (29). However, when flexible hinge regions in the neck are removed by mutagenesis, the microtubule gliding speed decreases at high motor density.

The reduced speed of our chimeras at high motor density could be due to a similar lack of flexibility at the lever arm-rod junction, because the structure formed by the first ~70 amino acids of the Myo4p rod is unknown.

The Globular Tail Affects Activity—A striking observation was that the actin-activated ATPase activity of the construct lacking the globular tail was higher than that of the full-length chimera, implying that the globular tail is involved in inhibiting activity. In the single-molecule assay, processive runs were less frequent for the full-length chimera than for ΔGT, whereas in the motility assay, the activity of the full-length and ΔGT chimeras were the same. Very similar observations were made when mouse myosin Va was compared with a truncated HMM construct; the full-length molecule had lower actin-activated ATPase and fewer processive runs than the truncated HMM construct, but actin movement in the motility assay was the same (22, 34). For myosin Va, these observations were accounted for by the fact that myosin Va assumes a folded, inhibited state in solution that is stabilized by a motor domain-globular tail interaction, and this interaction is disrupted when myosin Va binds to the nitrocellulose “cargo” in a motility flow cell (22). An inhibitory interaction involving the globular tail of Myo4p is suggested by our results. However, further studies are needed to establish if this regulation occurs in the native Myo4p molecule and, if so, what the exact nature of the interaction is. Biologically, it would be advantageous for molecules that are not complexed to cargo to be inactive.

Cargo Transport in Vivo—How many motors are likely to be used when Myo4p transports its cargo in vivo? A processive motor is essential only if a cargo has a single motor molecule bound. Even in higher organisms, such as Xenopus, more than 60 myosin Va motors are estimated to be present on one melanosome (35). ASH1 mRNA, a cargo of Myo4p, has four zip codes (cis-acting localization elements), each of which can bind a molecule of She2p (30, 36). The mRNA can therefore bind four high duty cycle motors, a situation that is closely mimicked by our multiple-motor experiment (Fig. 7a) (37). However, not all mRNAs have more than one zip code, and a single zip code is sufficient to localize ASH1 mRNA to the bud tip (38, 39). In this case, one could postulate that the Myo4p-She3p complex might be functionally dimerized in vivo, perhaps by its homodimeric binding partner She2p. If each subunit of She2p can bind one She3p, it would place two Myo4p-She3 complexes in close proximity (Fig. 7b). This situation is mimicked by our constructs dimerized with a leucine zipper.

Both myosin VI and the kinesin Unc104/KIF1A are highly processive motors as dimers and have been shown to undergo a reversible transition from monomer to dimer. Myosin VI purified from cells is monomeric (40), but upon increasing the local concentration by clustering the monomers on actin or cargo, myosin VI dimerizes (26). Similarly, KIF1A exists primarily as a monomer below 1 μM, whereas it is primarily dimeric above ~5 μM (41). Whether She2p (or other proteins) can act as a template to bring together two Myo4p-She3p complexes to create a functional two-headed motor is at present unknown.

Even for mRNAs that contain only one zip code, it is probable that a single mRNA is not carried to the bud in isolation. Although the exact composition of the translocation complex is unclear, it appears to include multiple mRNAs and proteins (17). Since only one particle is observed in each cell, it must include all copies of ASH1 mRNA that are transported to the bud. It might also include other mRNAs that are also localized to the bud tip, each of which contains at least one zip code capable of binding She2p and thus Myo4p (11). If this is the case, then there will be many binding sites for Myo4p, because there are multiple cargoes, thus enabling multiple motor transport. A large translocation particle might indeed require multiple motors to travel from mother to daughter cell through the viscous cytoplasm. Additionally, transport in yeast occurs along actin cables and not a single actin filament, providing more binding sites for multiple Myo4p motors.

Conclusion—Concurrent with our study, a complementary study of native Myo4p purified from yeast reached many of the same basic conclusions (27). The authors found that She3p co-purified with Myo4p when isolated from yeast, that the Myo4p-She3p complex was a single-headed motor, and that Myo4p-She3p was not processive. The similar findings of these two studies clearly demonstrate that the Myo4p rod is responsible for these properties. Because our myosins were expressed in Sf9 cells, we had considerably more protein than is available from yeast expression and thus were able to perform biochemical assays not possible for Dunn et al. (27). We used analytical ultra-

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centrifugation to obtain molecular weights and show that the Myo4p rod does not dimerize with itself, whereas Dunn et al. (27) used cross-linking and sucrose gradients. In addition, we localized the primary She3p binding site to the rod region and showed that it binds in a 1:1 molar ratio. The ΔGT construct showed that the rod is capable of dimerizing at micromolar protein concentration but is prevented from doing so by binding of She3p and, to a lesser extent, by the globular tail. Finally, we show that single monomeric motors are nonprocessive but that both multiple motors and dimerized constructs are able to support continuous cargo transport along actin filaments.

While this paper was under review, Heuck et al. (42) used analytical ultracentrifugation to show that a bacterially expressed construct containing the Myo4p rod and globular tail is monomeric. We showed that in the absence of She3p, the globular tail inhibits dimerization of the rod and agree with this finding. From these and other data, Heuck et al. (42) inferred that native Myo4p in the absence of She3p is also monomeric but can dimerize when in complex with She3p to form a two-headed translocation complex. Note, however, that they only measured the molecular weight in the absence of She3p. In contrast, our results suggest that She3p binding to the rod results in a single-headed motor complex and that if dimerization occurs in the cell, it would require another protein, such as the homodimeric downstream binding partner She2p.

With the knowledge gained about the properties of the Myo4p motor in vitro, experiments can be designed to better understand the nature of Myo4p transport in vivo. Is it accomplished by multiple monomers of the Myo4p-She3p complex? Can the downstream homodimeric binding partner She2p allow two molecules of Myo4p-She3p to form a functional dimer when bound to a zip code? Do multiple mRNAs in the translocation complex ensure that multiple motors are present even when an mRNA has only one zip code? How and if the activity of Myo4p is regulated also remains to be understood.

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