Two single-headed myosin V motors bound to a tetrameric adapter protein form a processive complex

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yo4p, one of two class V myosins in budding yeast, continuously transports messenger RNA (mRNA) cargo in the cell but is nonprocessive when characterized in vitro. The adapter protein She3p tightly binds to the Myo4p rod, forming a single-headed motor complex. In this paper, we show that two Myo4p–She3p motors are recruited by the tetrameric mRNA-binding protein She2p to form a processive double-headed complex. The binding site for She3p was mapped to a single α helix that protrudes at right angles from She2p. Processive runs of several micrometers on yeast actin–tropomyosin filaments were observed only in the presence of She2p, and, thus, motor activity is regulated by cargo binding. While moving processively, each head steps ~72 nm in a hand-over-hand motion. Coupling two high-duty cycle monomeric motors via a common cargo-binding adapter protein creates a complex with transport properties comparable with a single dimeric processive motor such as vertebrate myosin Va.

Introduction

A feature once thought to be a hallmark of all class V myosins was their ability to move processively, that is, to take multiple steps on actin tracks without dissociating. This concept arose from the fact that myosin Va, the most well-studied class V myosin from vertebrates, steps along actin for several micrometers, a feature well suited for transporting cargo in the cell. However, several class V myosins involved in intracellular cargo transport have been characterized as nonprocessive under in vitro conditions. These include human myosin Vc (Takagi et al., 2008; Watanabe et al., 2008), Drosophila melanogaster myosin V (Tóth et al., 2005), and both class V myosins (Myo2p and Myo4p) from the budding yeast Saccharomyces cerevisiae (Reck-Peterson et al., 2001; Dunn et al., 2007; Hodges et al., 2008).

How can nonprocessive motors function as cargo transporters? Processivity is only necessary to achieve long continuous runs if a single motor is attached to its cargo. Multiple nonprocessive motors may collectively be as effective at transport as a single processive one. Alternatively, most in vitro studies have been performed using bare actin filaments in the absence of any bound cargo, and, thus, motors characterized as nonprocessive might be processive when assayed under more physiologically relevant conditions. Here, we focus on understanding potential mechanisms by which Myo4p, one of the two nonprocessive class V myosins from budding yeast, can continuously transport and asymmetrically localize >20 different mRNAs (Shepard et al., 2003; Jambhekar et al., 2005) to the bud tip via actin cables. Asymmetric localization of mRNA is a widely used mechanism to allow cells to spatially and temporally control protein function by determining their sites of synthesis.

Myo4p has some unusual features for a class V myosin. Although it has a high duty cycle motor domain and a long lever arm (Krementsova et al., 2006), it is single headed and thus cannot move processively as a single motor (Fig. 1 A; Dunn et al., 2007; Hodges et al., 2008). Instead of forming an α-helical coiled-coil homodimer, as do all other class V myosin heavy chains, the rod region of Myo4p tightly binds to the cargo
Results

She2p is a tetramer

She2p links the Myo4p–She3p motor complex to its mRNA cargo. The crystal structure of She2p showed that it was a homodimer related by a twofold axis of symmetry (Fig. 1 B; Niessing et al., 2004). The She2p monomer consists of a five–α helix bundle with a basic helical hairpin motif that binds mRNA. Analysis of She2p by sedimentation velocity in the analytical ultracentrifuge showed a pronounced tendency to form multiple higher oligomers, even in the presence of DTT, as also observed by others (Fig. 1 C; Müller et al., 2009). Because higher-order oligomers would complicate interpretation of our further studies, we adopted some of the strategy used to crystallize She2p, namely mutation of four Cys residues (14, 68, 106, and 180) to Ser (Fig. 1 B). This effectively abolished the tendency of She2p to aggregate via formation of disulfide bonds (Fig. 1 C), resulting in a homogeneous species that sedimented at 5.4 S (Table I). This homogeneous She2p construct (called wild type* [WT*]) was used for all experiments and is the backbone for subsequent mutations.

The ability of She2p WT* to functionally replace native She2p in living she2Δ yeast cells was tested. The most well-studied mRNA in budding yeast is ASH1, which is moved by Myo4p to the bud tip to repress mating-type switching in the daughter cell (Haarer et al., 1994; Sil and Herskowitz, 1996; Long et al., 1997; Takizawa et al., 1997). Mutations in any of the five genes SHE1–SHE5 cause defects in ASH1 mRNA transcript localization. Actively budding yeast cells that contained one fluorescent ASH1 mRNA particle were scored according to whether the particle was localized in the bud tip (correct localization) or in the mother cell (incorrect localization; Fig. 2 A).

She3p might form a hetero–coiled-coil, which would explain the inability of Myo4p to form a homodimer. Although most class V myosins have multiple adapter proteins to allow binding to a variety of different cargoes, Myo4p uses She3p as the sole adapter protein for its two types of cargo, mRNA and cortical ER (Estrada et al., 2003; Shepard et al., 2003; Jambhekar et al., 2005; Schmid et al., 2006). Incorporating She3p as a tight-binding subunit of Myo4p thus makes biological sense.

Here, we begin to increase the complexity of the characterization of the Myo4p–She3p motor complex in vitro by introducing the mRNA-binding protein She2p. She2p is the middleman that binds to both the motor (Myo4p–She3p) and the cargo (mRNA). We show by EM that She2p recruits two Myo4p–She3p motors. Unlike a single Myo4p–She3p motor, this complex supports long-range continuous transport on actin. Surprisingly, the two motors linked via She2p show hand-over-hand stepping indistinguishable from vertebrate myosin Va, which is dimerized by an α-helical coiled-coil. The ability to show processive motion only when bound to the cargo adapter protein She2p provides an elegant mechanism by which this motor can be regulated. The results suggest that motors that have been characterized as nonprocessive in vitro may achieve the continuous motion expected of a cargo transporter under conditions that more closely mimic the cellular situation.

adapter protein She3p. The two proteins copurify, and She3p is, in essence, a subunit of the motor complex (Figs. 1 A and S1 A; Dunn et al., 2007; Hodges et al., 2008). Based on the fact that both proteins have coiled-coil motifs, the rod of Myo4p and
Transformation of the she2Δ cells with WT She2p showed 97% of the particles being correctly localized to the bud tip compared with 96% for She2p WT* (Fig. 2 B). Mutation of the four Cys residues to Ser thus did not affect the cellular function of She2p.

The molecular mass of the 5.4 S She2p WT*, obtained by sedimentation equilibrium in the analytical ultracentrifuge, was 98.7 ± 3.9 kD (Table I). Given that the monomer molecular mass of She2p is 28 kD, this result is most consistent with She2p being a tetramer, which is in agreement with recent results from two other groups (Müller et al., 2009; Chung and Takizawa, 2010). The dimeric She2p structure observed by crystallography (Niessing et al., 2004) is therefore not the state of oligomerization that exists in solution.

**Table I. Oligomeric state of WT and mutant She2p**

<table>
<thead>
<tr>
<th>She2p</th>
<th>Sedimentation value</th>
<th>MM</th>
<th>Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT*</td>
<td>5.4</td>
<td>98.7 ± 3.9</td>
<td>Tetramer</td>
</tr>
<tr>
<td>L130Y</td>
<td>4.1</td>
<td>54.4 ± 1.0</td>
<td>Dimer</td>
</tr>
<tr>
<td>S120Y</td>
<td>4.0</td>
<td>51.7 ± 1.3</td>
<td>Dimer</td>
</tr>
<tr>
<td>Δhelix</td>
<td>5.4</td>
<td>ND</td>
<td>Tetramer</td>
</tr>
</tbody>
</table>

The calculated molecular mass (MM) of a She2p monomer is 28 kD.

Figure 2. *Ability of various She2p constructs to correctly localize ASH1 mRNA particles in budding yeast.* (A) Representative images of correctly and incorrectly localized ASH1 mRNA. Correctly localized mRNA is found in the bud, whereas particles that are not transported remain in the mother cell. (left) Differential interference contrast [DIC]. (middle) Epifluorescence to visualize GFP-labeled ASH1 mRNA. (right) Merged images. (B) Percentage of correctly localized ASH1 mRNA particles for various She2p constructs. The indicated constructs were transformed into a she2Δ strain. Constructs lacking She2p do not localize ASH1 mRNA. WT* is the WT construct with four Cys residues (14, 68, 106, and 180) mutated to Ser. All other constructs are based on the WT* backbone. Addition of a fluorescent protein to either the N or C terminus of She2p had no effect on cellular function. Data are shown for a C-terminal fluorescent protein, but the data are identical for YFP at the N terminus. The negative control (neg) is a plasmid lacking an insert. The number of cells counted is indicated above each bar. Data were derived from at least two independent transformations per construct.

Deletion of the protruding α helix on She2p abolishes She3p binding

The binding site on She2p for She3p has not been previously mapped. The She2p structure shows a prominent α helix protruding at right angles from the middle of each monomer (Fig. 1 B). This helix, and its connection to the five-α helix bundle, is conserved when compared with nine other yeast species (Niessing et al., 2004). Based on this observation and given that single α helices are generally not stable in solution except when bound to a partner, we speculated that this helix in She2p constitutes a major part of the binding site for She3p. When residues V174–K179 of She2p were deleted (She2p-Δhelix), Myo4p–She3p did not bind, as assessed by an actin-pelleting assay (Fig. 4), nor was it pulled down with the FLAG-tagged Δhead–She3p complex (Fig. S1 B). However, deletion of these residues had no effect on the homogeneity or S value of She2p (Table I). When the She2p-Δhelix construct was transformed into she2Δ yeast cells, only 8% of the particles was correctly localized, similar to the value obtained with a negative control (Fig. 2). No mRNA localization is seen when the connection between the motor and She2p is disrupted (Bookwalter et al., 2009), and, thus, we conclude that this helix constitutes all or part of the binding site for She3p.

**Point mutants of She2p disrupt tetramer formation**

She2p with various point mutations has been tested for mRNA binding in vitro and mRNA localization in situ (Niessing et al., 2004). Two of these mutations also affect the oligomeric state of She2p. She2p(S120Y), located at the interface of the
binds to the Myo4p–She3p complex (Fig. 4). When tested in vivo, this mutant supported correct localization of mRNA to the same high extent as WT and WT* She2p (Fig. 2). Two Myo4p–She3p motors bind to a She2p tetramer and form a processive complex EM was used to determine the number of Myo4p–She3p motors that bind to a She2p tetramer. Metal-shadowed images of Myo4p–She3p alone showed an elongated structure with a globular domain at one end, which is presumably the myosin motor domain (Fig. 5). Two-headed structures were not observed. In contrast, when Myo4p–She3p was incubated with She2p, 25 ± 7% of the images (mean ± SD; n = 186; five fields) showed a V-shaped image, consistent with two Myo4p–She3p motors bound to a She2p tetramer. In the majority of images, the only contact between the two motors appears to be at the base where She3p binds to She2p. We conclude that a She2p tetramer recruits two Myo4p–She3p motor complexes. Two-headed structures were not observed with either the S120Y or the L130Y mutant She2p dimers.

Two Myo4p–She3p motors bind to a She2p tetramer and form a processive complex EM was used to determine the number of Myo4p–She3p motors that bind to a She2p tetramer. Metal-shadowed images of Myo4p–She3p alone showed an elongated structure with a globular domain at one end, which is presumably the myosin motor domain (Fig. 5). Two-headed structures were not observed. In contrast, when Myo4p–She3p was incubated with She2p, 25 ± 7% of the images (mean ± SD; n = 186; five fields) showed a V-shaped image, consistent with two Myo4p–She3p motors bound to a She2p tetramer. In the majority of images, the only contact between the two motors appears to be at the base where She3p binds to She2p. We conclude that a She2p tetramer recruits two Myo4p–She3p motor complexes. Two-headed structures were not observed with either the S120Y or the L130Y mutant She2p dimers.

Total internal reflection fluorescence (TIRF) microscopy was used to determine whether the complex of two Myo4p–She3p motors bound to She2p can move processively on actin (Fig. 6 A).
Two single-headed myosin Vs move processively • Krementsova et al.

[561x25]635

with an intensity more than 10 times that of the mean particle were also observed to move processively but at a much slower speed (0.36 ± 0.19 µm/s in 1 mM MgATP; \( n = 6 \)). Thus, the vast majority of moving particles are composed of tetrameric She2p with two Myo4p–She3p motors bound.

Processivity of Myo4p–She3p–She2p is seen with both yeast actin–tropomyosin and bare skeletal actin, but the run frequency is 11.2-fold higher on yeast actin–tropomyosin. For comparison, the run frequency of processive dimeric mammalian myosin Va on bare skeletal actin is three orders of magnitude higher than that of the Myo4p complex. Thus, it is likely that not all of the Myo4p–She3p is complexed with She2p at nanomolar concentrations, and more stable binding may require mRNA.

When the tetrameric mutant lacking the protruding \( \alpha \) helix was tested in this assay, no processive runs were observed, which is consistent with results from our in vitro and in vivo characterization of this mutant. Occasional processive runs were observed with the two dimeric She2p point mutants (S120Y and L130Y), but the frequency of events was greatly reduced relative to WT*–She2p (Table II). Although the point mutations shift the equilibrium from tetramer to dimer, it is possible that a small fraction of tetramers remains for both mutants. In addition, She2p(S120Y) showed a tendency to form larger nonspecific aggregates, likely accounting for the observed processive runs with this mutant.

The Myo4p–She3p–She2p complex steps hand over hand

To determine whether the two Myo4p heads are coordinated during processive stepping, streptavidin-coated quantum dots (Qdots) were bound to an N-terminally biotinated Myo4p construct. An equimolar mixture of red (655 nm) and green (565 nm) Qdots was used to label the motor domains (Fig. 7 A). No processive runs were observed in the absence of She2p. She2p was then added to recruit two Myo4p–She3p motors per She2p tetramer. Most complexes were labeled with only one Qdot, allowing us to track the motion of one of the two motor domains. A histogram of step sizes showed a mean step of 71.8 ± 18 nm (Fig. 7 B, inset), equal to twice the 36-nm semirepeat of F-actin. This implies that the center of mass moves 36 nm each step, which is very similar to that of the more well-studied dimeric vertebrate myosin Va molecule. The stepping pattern of two representative singly labeled complexes is shown in Fig. S3. A few complexes contained a different colored Qdot on each head. The stepping pattern of the dual-labeled motor complex

Table II. Frequency of processive runs by WT and mutant She2p

<table>
<thead>
<tr>
<th>She2p</th>
<th>Number of events</th>
<th>Event frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT*</td>
<td>59</td>
<td>56.4</td>
</tr>
<tr>
<td>S120Y</td>
<td>17</td>
<td>10.7</td>
</tr>
<tr>
<td>L130Y</td>
<td>5</td>
<td>1.3</td>
</tr>
<tr>
<td>( \Delta )helix</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The values in the second column were obtained with chicken skeletal actin. Event frequency for WT* is 11.2 times higher on yeast actin–tropomyosin.

The Myo4p–She3p–She2p complex steps hand over hand

To follow motion of the ternary complex, a She2p-YFP fusion construct was created. Control experiments in budding yeast confirmed that the YFP tag on She2p did not affect the ability of the motor complex to bind because >98% correct localization of ASH1 mRNA was obtained in she2Δ yeast cells transformed with She2p-YFP (Fig. 2).

We previously showed that Myo4p–She3p is nonprocessive in the absence of She2p (Hodges et al., 2008), which is in agreement with other laboratories (Reck-Peterson et al., 2001; Dunn et al., 2007). When Myo4p–She3p was mixed with She2p–YFP, YFP particles were observed to move processively along single filaments of yeast actin–tropomyosin (Tpm1p) for distances of up to 6 µm (Fig. 6 [B and C] and Video 1). The mean speed was 1.50 ± 0.49 µm/s (mean ± SD) in 1 mM MgATP, consistent with previous Myo4p ensemble motility measurements (Reck-Peterson et al., 2001; Dunn et al., 2007; Hodges et al., 2008). A mean run length of 1.38 µm was observed by fitting the distribution of measured run lengths (\( x \)) to a single exponential equation, \( y = Ae^{-\lambda x} \), in which \( \lambda \) is the mean run length, and \( A \) is a constant (Fig. 6 C). The intensity of the majority of particles was consistent with approximately four YFPs from the tetrameric She2p (Fig. S2). Occasionally, aggregates

![Figure 6.](image_url)
showed a hand-over-hand stepping pattern, with the lead and trailing head changing position with each step on actin, which is again very similar to myosin Va (Fig. 7 B).

**Discussion**

Here, we show that Myo4p–She3p, the single-headed class V myosin from budding yeast, supports processive motion on actin only when bound to the tetrameric adapter protein She2p. This is possible because the motor domain of Myo4p has a high-duty cycle (Hodges et al., 2008) and because two Myo4p–She3p motors are recruited per She2p tetramer. The two motors are coupled only by virtue of being bound to the same She2p tetramer in contrast to mammalian myosin Vs, which are dimerized via an α-helical coiled-coil. Given this fundamental difference, it was surprising that the two Myo4p–She3p motors bound to She2p take ~72-nm steps in a hand-over-hand fashion, the same size and stepping pattern exhibited by myosin Va (Yildiz et al., 2003; Warshaw et al., 2005). The main determinants of the common step size of myosin Va and Myo4p–She3p are the long neck or lever arm that binds six CaMs and/or light chains and the pseudorepeat of the actin helix rather than the exact geometry by which the two heads are joined.

The long run lengths of myosin Va are believed to be possible because of gating, meaning that the two heads communicate to keep their kinetic cycles out of phase. This communication is thought to occur via strain transmitted through the two lever arms, which predominantly acts to prevent premature dissociation of the leading head from actin (Purcell et al., 2005; Veigel et al., 2005; Oguchi et al., 2008). Electron microscope images are consistent with the idea that myosin Va is distorted and under tension when both heads are attached to actin (Walker et al., 2000; Oke et al., 2010). Although we cannot rule out that the two Myo4p–She3p motors bound to She2p form a four-stranded coiled-coil, it appears likely that any strain between Myo4p heads is mediated indirectly via She2p. It is unclear whether the two Myo4p heads are gated, but the ~1.4-µm run length of the Myo4p–She3p–She2p complex compares favorably with run lengths of 0.8–1.8 µm determined for myosin Va (Baker et al., 2004; Hodges et al., 2007; Ali et al., 2008). It has recently been argued that the detailed tuning of structure and intramolecular communication that ensures gating is not essential for processivity, provided that the motor heads have a sufficiently high-duty ratio and that multiple motor heads simultaneously interact with the actin network (Elting et al., 2011). The 1.4-µm run length of Myo4p corresponds to 39 steps of 36 nm. According to a simple two-state model, two heads cycling independently with no gating could achieve this run length if they had a duty ratio of ~87% (Veigel et al., 2002). However, gating undoubtedly improves the efficiency of the motor by preventing the wasting of ATP on unproductive cycles and plays a role in minimizing backward motion under load by preventing the front head from releasing from the actin filament.

**Features of She2p**

Our conclusion that She2p is a tetramer, which is in agreement with two other groups (Chung and Takizawa, 2010; Heuck et al., 2010), is inconsistent with crystallographic experiments, which concluded that She2p is dimeric (Niessing et al., 2004). To reconcile the crystallographic experiments with the solution experiments, one must assume either that the N- and C-terminal residues that were truncated to obtain crystals are necessary for tetramerization or that the crystallization conditions abolished interactions necessary for tetramer formation while retaining the large and presumably stronger dimer interface.

Here, we show that the conserved helix that protrudes at right angles from the She2p structure provides all or part of the binding site for She3p (Fig. 1 B). Müller et al. (2011) also demonstrated that a mutant similar to our Δhelix does not bind She3p but assigned the primary function of this region to mRNA binding based on UV cross-linking. The major mRNA-binding site on She2p has been mapped to a basic helical hairpin RNA–binding motif consisting of two antiparallel α helices separated by a loop (residues 36–63; Fig. 1 B, tan-colored region; Niessing et al., 2004). The only other conserved
region of She2p that has not been assigned a function is its C terminus, and that is dispensable for She3p binding (Müller et al., 2011). Collectively, it is thus likely that the primary role of the protruding helix on She2p is to bind She3p. Because only two motors are recruited per She2p tetramer, it is possible that two of the protruding helices are involved in binding one Myo4p–She3p motor.

Two point mutants convert the She2p tetramer into a dimer. S120Y lies at the interface of the crystallographic dimer, whereas L130Y lies at the interface of the proposed elongated tetrameric interface (Müller et al., 2009). Mutation of these residues might produce different dimers, i.e., a longitudinal versus a lateral dimer. Indeed, the two mutations had very different effects on ASH1 mRNA localization in the cell. She2p(L130Y) was indistinguishable from WT, whereas She2p(S120Y) showed no correct ASH1 mRNA localization. The lack of ASH1 mRNA localization with She2p(S120Y) is consistent with its weak binding to both Myo4p–She3p and mRNA (Niessing et al., 2004), which likely compromises the coupling between motor and cargo (Table III). But how can the She2p(L130Y) mutant function like WT in the cell, as it did not support processive motion in vitro? One possibility is that multiple motors are recruited when multiple mRNAs are cotransported (Lange et al., 2008). Alternatively, it is possible that the presence of mRNA in the cell acts to stabilize some or all of the She2p(L130Y) as a tetramer.

Role of mRNA in complex formation
Our data show that baculovirus-expressed Myo4p–She3p can bind She2p at nanomolar concentration in the absence of mRNA. However, the relatively low run frequency compared with dimeric mammalian myosin Va suggests that not all of the Myo4p–She3p is likely to be bound to She2p in the absence of mRNA. This observation is consistent with several other lines of evidence. A 20-S complex isolated from budding yeast contained Myo4p, She3p, and She2p only when intact mRNA was present (Chung and Takizawa, 2010). RNase treatment caused the complex to dissociate based on sucrose gradient sedimentation assays. Another recent study showed that mRNA substantially stabilizes the She3p–She2p interaction, caused in part by the ability of She3p to directly bind to mRNA. It was concluded that the mRNA itself triggers joining of all components into the high-specificity mature transport complex (Müller et al., 2011). An attractive feature of this idea is that only She2p that has bound and escorted ASH1 mRNA out of the nucleus would form a high affinity and specific complex with cytoplasmic Myo4p–She3p. We are currently examining the single molecule properties of Myo4p–She3p–She2p in the presence of ASH1 mRNA and speculate that the number of processive events will increase substantially in the presence of mRNA.

Strategies for single-headed motors to move processively
Other mechanisms by which single-headed motors can achieve processive motion within the cell have been proposed. Full-length myosin VI isolated from cells or expressed in baculovirus is monomeric (Sweeney and Houdusse, 2010). It was suggested that when myosin VI is bound to the adapter proteins optineurin or Dab2, an internal dimerization sequence in the rod is exposed, which triggers coiled-coil formation (Phichith et al., 2009). Interestingly, even the monomeric adapter protein Dab2 is capable of dimerizing myosin VI because it contains two discrete binding sites for the tail (Yu et al., 2009). A preexisting oligomer is thus not required for dimerization. Myo4p–She3p differs from myosin VI in that it is not internally dimerized but simply joined via She2p.

Another mechanism involves clustering of monomeric motors on membrane vesicles, which then favors internal dimerization. Monomeric myosin VIIA required forced dimerization to transport cargo to the filopodial tip (Sakai et al., 2011). Because the adapter proteins MyRip/Rab27a did not directly dimerize myosin VIIA, it was proposed that the motors dimerize when closely clustered on the vesicle. Deletion of a predicted short coiled-coil domain did not diminish localization, and, so, the dimerization domain in myosin VIIA is unknown.

An alternative mechanism for monomeric motors to achieve long-range transport is to work in ensembles. The motion of endosome-sized nanospheres coated with multiple monomeric myosin VI motors on a skinned keratocyte lamellipodium was tracked (Sivaramakrishnan and Spudich, 2009). Multiple monomers interacting with multiple actin filaments could cooperate to move continuously for long distances under low load. Similarly, we previously showed that robust continuous movement was observed when multiple (approximately three to four) monomeric Myo4p–She3p motors were bound to a Qdot cargo (Hodges et al., 2008). Here, we show that only two Myo4p–She3p motors are necessary to support long-range processive transport when coupled by She2p.

### Table III. Summary of properties of WT and mutant She2p constructs

<table>
<thead>
<tr>
<th>She2p</th>
<th>Oligomeric state in solution</th>
<th>Binds to She3p in vitro</th>
<th>Processive</th>
<th>Binds to mRNA in vitro</th>
<th>Correct localization of ASH1 mRNA in yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT*</td>
<td>Tetramer</td>
<td>++++</td>
<td>+</td>
<td>++++ a</td>
<td>+</td>
</tr>
<tr>
<td>S120Y</td>
<td>Dimer</td>
<td>+</td>
<td>–</td>
<td>+ a</td>
<td>–</td>
</tr>
<tr>
<td>L130Y</td>
<td>Dimer</td>
<td>+++</td>
<td>–</td>
<td>+ a</td>
<td>+</td>
</tr>
<tr>
<td>Δhelix</td>
<td>Tetramer</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

aRelative ability of 1 µM She2p to bind to mRNA using filter-binding experiments (Niessing et al., 2004).

bValues from mRNA filter-binding experiments using the ASH1 mRNA E3 zip code element (Müller et al., 2009).

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Implications for cellular cargo transport

Our findings imply that a mechanism for regulating cargo motility by monomeric motors is to help or hinder their transition to a functional dimer. In budding yeast, She2p interacts with ASH1 mRNA in the nucleus and shuttles it into the cytoplasm (Fig. 8; Du et al., 2008; Shen et al., 2009). Based on our studies, once in the cytoplasm, the She2p–mRNA complex should be able to recruit two Myo4p–She3p motors per zip code, the localization element on the mRNA that binds She2p. Although She2p can bind to Myo4p–She3p in the absence of mRNA, in the cell, there needs to be a checkpoint to ensure that She2p is coupled to mRNA before it encounters the motor. This is likely facilitated by the fact that She3p is cytoplasmic, but She2p binds to mRNA in the nucleus and then escorts it into the cytoplasm. A recent study suggests an additional level of control, that once in the cytoplasm, the She2p–mRNA complex should be able to recruit two Myo4p–She3p motors per zip code, the localization element on the mRNA that binds She2p. Although She2p can bind to Myo4p–She3p in the absence of mRNA, in the cell, there needs to be a checkpoint to ensure that She2p is coupled to mRNA before it encounters the motor. This is likely facilitated by the fact that She3p is cytoplasmic, but She2p binds to mRNA in the nucleus and then escorts it into the cytoplasm. A recent study suggests an additional level of control, that once in the cytoplasm, the She2p–mRNA complex should be able to recruit two Myo4p–She3p motors per zip code, the localization element on the mRNA that binds She2p. Although She2p can bind to Myo4p–She3p in the absence of mRNA, in the cell, there needs to be a checkpoint to ensure that She2p is coupled to mRNA before it encounters the motor. This is likely facilitated by the fact that She3p is cytoplasmic, but She2p binds to mRNA in the nucleus and then escorts it into the cytoplasm. A recent study suggests an additional level of control, that once in the cytoplasm, the She2p–mRNA complex should be able to recruit two Myo4p–She3p motors per zip code, the localization element on the mRNA that binds She2p. Although She2p can bind to Myo4p–She3p in the absence of mRNA, in the cell, there needs to be a checkpoint to ensure that She2p is coupled to mRNA before it encounters the motor. This is likely facilitated by the fact that She3p is cytoplasmic, but She2p binds to mRNA in the nucleus and then escorts it into the cytoplasm. A recent study suggests an additional level of control, that once in the cytoplasm, the She2p–mRNA complex should be able to recruit two Myo4p–She3p motors per zip code, the localization element on the mRNA that binds She2p. Although She2p can bind to Myo4p–She3p in the absence of mRNA, in the cell, there needs to be a checkpoint to ensure that She2p is coupled to mRNA before it encounters the motor. This is likely facilitated by the fact that She3p is cytoplasmic, but She2p binds to mRNA in the nucleus and then escorts it into the cytoplasm.
yeast CaM, 25 µg/ml Mlc1p, and protease inhibitors (0.5 mM 4-(2-acetoamidoethyl)benzenesulfonyl fluoride, 5 µg/ml leupeptin, and 0.78 mg/ml benzamidine). The cells were lysed by sonication, and the lysate was centrifuged for 15 min at 250,000 g in the presence of 2 mM MgATP. After incubation with FLAG affinity resin (Sigma-Aldrich) for 30 min, the resin was sedimented for 5 min at 1,000 rpm and washed with buffer (10 mM imidazole, pH 7.4, 0.3 M NaCl, and 1 mM EGTA). Bound protein was eluted using a 1-µg/ml solution of FLAG peptide in the same buffer. Fractions were pooled, concentrated in 50% glycerol, and stored at −20°C.

All She2p variants were expressed as GST fusion proteins in E. coli Bl21(DE3). A 5-µl overnight culture grown in Lennox broth was diluted 50 times into enriched media and grown for 3.5 h at 37°C. After induction (with 1 mM IPTG), the cells were grown overnight at 25°C. The cells were lysed by sonication in PBS with protease inhibitors and loaded onto a 20-ml glutathione Sepharose 4B column (GE Healthcare). The column was washed with 5 vol PBS and drained. 90 U thrombin (Roche Diagnostics, Inc.) in 5 ml PBS was added to the column, and the slurry was incubated for 1 h at room temperature with occasional mixing. The column was drained and washed with 25 ml PBS. 1 mM DTT was added following by 2 µg/ml leupeptin to inactivate the thrombin. Thrombin was removed by passing the mixture over a benzamidine Sepharose 6B column (GE Healthcare). The protein was concentrated in a 50-µl Centricon (Millipore) to ~5 µl and stored in 50% glycerol with 0.2 M NaCl, 10 mM imidazole, pH 7.5, 0.2 mM CaCl2, and 1 µM DTT.

Chicken skeletal actin was prepared from acetone powder (Porred and Spudich, 1982). Chicken actin was expressed using the baculovirus/insect cell expression system. SF9 cells were infected with recombinant baculovirus encoding yeast actin and harvested 72 h later. Cells were lysed using 1 M Tris-HCl, pH 7.5, 0.6 M KCl, 0.5 mM MgCl2, 0.5 mM NaATP, 1 mM DTT, 4% Triton X-100, 1 µg/ml Tween 20, 0.5 mM AEBSF, 0.5 mM tosyllysine chloromethyl ketone, and 5 µg/ml leupeptin and stirred for 1.5 h at 4°C. The lysate was clarified for 1 h at 177,700 g. The supernatant was subjected to a 20°C, 20,000 g centrifugation, and the pellet was assessed by SDS-PAGE.

For Mlc1p, the clarified supernatant was incubated with 3 µl FLAG resin for 1 h. The resin was washed with the same buffer as above but without NaATP and DTT. The supernatant was incubated with 3 ml FLAG resin for 1 h. The resin was washed with the same buffer as above but without NaATP and DTT. The supernatant was incubated with 3 ml FLAG resin for 1 h. The resin was washed with the same buffer as above but without NaATP and DTT. The supernatant was incubated with 3 ml FLAG resin for 1 h. The resin was washed with the same buffer as above but without NaATP and DTT.

Actin was then eluted by dialysis against 20 mM Hepes, pH 7.0, 0.3 M NaCl, 1 mM DTT, 1 mM EGTA, and 1 mM NaN3. The data were analyzed with Origin software provided with the X-Cite 120 (EXFO) and a 14-bit camera (CoolSNAP HQ2; Photometrics) were used. Images were processed with NIH Elements (NIKON) and ImageJ (National Institutes of Health) software. Actively budding yeast were scored according to the location and number of fluorescent particles seen. Actively budding yeast cells that contained one fluorescent mRNA particle were scored according to whether the particle was located in the bud tip (correct localization) or the mother (incorrect localization).

Analytical ultracentrifugation

An analytical ultracentrifuge (Optima XL-I; Beckman Coulter) was used to determine the sedimentation coefficients of the expressed constructs. Sedimentation velocity runs were performed in the An-60 Ti rotor at 40,000 rpm and 20°C in 10 mM Hepes, pH 7.0, 0.3 M NaCl, 1 mM DTT, 1 mM EGTA, and 1 mM NaN3. 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 (Philo, 2000). Sedimentation equilibrium data were collected at 15,000 rpm and 4°C, 10 mM Hepes, pH 7.0, 0.3 M NaCl, 1 mM DTT, 1 mM EGTA, and 1 mM NaN3. The data were analyzed with Origin software provided with the Optima XL-I ultracentrifuge. For molecular mass calculations, the fit to the mean of three independent scans at equilibrium was determined for each protein sample, and the mean and SD of those values were calculated for each construct.

Actin-pelleting assay

0.3 µM Myo4p–She3p was mixed with various She2p constructs (~1.2 µM monomer) and incubated for 10 min in 10 mM imidazole, pH 7.5, 1 mM EGTA, 1 mM DTT, and 0.2 M NaCl. Both proteins were preclarified before mixing together. 2.4 µM actin was then added. The mixtures were pelleted for 20 min at 400,000 g. The supernatant was removed, and the pellet was washed twice with buffer. Protein content of the supernatant and pellet was assessed by SDS-PAGE.

FLAG resin pull-down assay

A truncated Myo4p construct lacking the motor domain and neck was coexpressed with She3p and assessed for binding to various She2p constructs. Both proteins were clarified at 400,000 g for 15 min before mixing. Myo4p–She3p and She2p (each at 1 µM) were mixed together in 250 µl and loaded onto 0.3-mL FLAG columns (Sigma-Aldrich). The columns were washed with 10 mM imidazole, pH 7.5, 1 mM EGTA, and 0.2 M NaCl, and bound proteins were eluted with 0.1 M MgCl2 in 10 mM imidazole and 1 mM DTT. Fractions were analyzed on SDS gels.

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Myo4p–She3p and She2p-WT* were dialyzed into 10 mM MOPS, pH 7.5, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, and 1 mM DTT. Myo4p–She3p and She2p were mixed at a molar ratio of 6:1, incubated for 30 min in

ASH1 mRNA reporter system

A two-plasmid system was used to follow ASH1 mRNA localization. The two plasmids pG14-MS2-GFP with LEU2 selection and YEplac195 lacZ-MS2-ASH1 with URA3 selection were gifts from R. Long (Medical College of Wisconsin, Milwaukee, WI; Bertrand et al., 1998). pG14-MS2-GFP expresses an MS2 coat protein-GFP under a constitutive promoter. The MS2-GFP chimera contains an NLS to confine unbound MS2-GFP to the nucleus. YEpplac195 lacZ-MS2-ASH1 expresses the ASH1 mRNA along with six stem loop–binding motifs for the MS2 coat protein under control of a galactose-inducible promoter. The URA3 marker in YEpplac195 lacZ-MS2-ASH1 was replaced with HIS3.

Visualization of transformed yeast

The mutant yeast strain K5477 (MATa ade2 his3 leu2 trp1 ura3 can1-100 she2::URA [W303a background]) was used. The yeast strain she2Δ was transformed with three plasmids. Two were required for the ASH1 reporter system. The third plasmid contained the various forms of She2p, including a WT used as a positive control and a plasmid without insert as the negative control. Transformants were grown on the appropriate complete synthetic medium lacking the nutrients needed to maintain the plasmids. To induce expression of the various versions of She2p along with the reporter mRNA, a single colony was grown overnight in the same synthetic medium containing 2% galactose as the sole carbon source. 4 h before visualization, the yeast cells were streaked in a fresh patch to ensure exponential growth when viewed and scored. The live cells were mounted on a slide in the appropriate liquid medium containing galactose and viewed with an inverted microscope (TE2000-E; Nikon) using a Plan Apo 60x oil objective lens and differential interference contrast and FITC filters. A fluorescence illuminator (X-Cite 120; EXFO) and a 14-bit camera (CoolSNAP HQ2; Photometrics) were used. Images were processed with NIH Elements (NIKON) and ImageJ (National Institutes of Health) software. Actively budding yeast were scored according to the location and number of fluorescent particles seen. Actively budding yeast cells that contained one fluorescent mRNA particle were scored according to whether the particle was located in the bud tip (correct localization) or the mother (incorrect localization).
ice, and then diluted in the same buffer containing 50% glycerol to a final concentration of 60–120 µg/ml. 5–10 µl of each sample was sprayed on a freshly split mica surface, dried for 2 h under vacuum, rotary shadowed with platinum at an angle of 7°, and replicated with carbon in a Balzers 410 freeze-fracture machine. Replicas were photographed in an electron microscope [410; Philips] operating at 60 kV at a magnification of 75,300. The negatives were digitally scanned at 1,000 pixels per inch. Images were obtained from areas at the edge of each droplet that showed distinct molecules and a clear background.

TIRF microscopy

For experiments in which run lengths were measured, motion was followed from the YFP signal on She2p. She2p–YFP was purified and added to the flow chamber at 30 nM. 0.2 µM Myo4p–She3p was added, resulting in final concentrations of 40 nM N-terminally biotinated Myo4p-She3p and 50 nM She2p–YFP. Nethylmaleimide–modified skeletal muscle myosin forms a strong and ATP-insensitive bond with actin and was used to attach the actin filaments to the coverslip.

Data were collected on an inverted microscope (Eclipse TI-U; Nikon) equipped with a 100× Plan Apo objective lens [1.49 NA] and auxiliary 1.5x magnification for through-the-objective TIRF microscopy. The YFP was excited with a 473-nm laser line. Images were obtained using a camera (XR/Turbo-Z; Stanford Photonics) running Piber Control software [v2.3.39]. The pixel resolution was 95.4 nm. Data were collected at 10–20 frames per second. Movement of She2p–YFP was tracked manually using ImageJ.

For experiments in which the stepping pattern of the Myo4p–She3p–She2p complex was determined, motion was followed from a Qdot attached to the N terminus of Myo4p. She2p (without YFP) was purified and added to the flow chamber at 400,000 g for 20 min before use. N-terminally biotinated Myo4p-She3p was mixed with red (655-nm emission) streptavidin Qdots [Invitrogen] at a ratio of five Qdots per Myo4p monomer. A similar solution was prepared using green (565-nm emission) Qdots, and both solutions were incubated on ice for 20 min. They were then mixed in a 1:1 ratio, and She2p was added, resulting in final concentrations of 40 nM N-terminally biotinated Myo4p-She3p and 160 nM She2p [monomer concentration]. Flow cells were prepared as described above. Alexa Fluor 594 phalloidin yeast actin–Tpm1p filaments or chicken skeletal muscle myosin (5-min incubation), 5× rinse with motility buffer, and, finally, Myo4p–She3p diluted in motility buffer with either 10 µM, 1 mM, or 2 mM MgATP. Motility buffer consists of 50 mM KCl, 25 mM imidazole, pH 7.4, 4 mM MgCl2, 1 mM EGTA, 50 mM DTT, 1 mM BSA, 0.2 mM/ml yeast CoA, 0.2 mg/ml Mic1p, an ATP-regenerating system (0.5 mM phosphoenolpyruvate and 100 U/ml pyruvate kinase), and an oxygen-scavenging system (0.1 mM glucose, 0.1 mg/ml glucose oxidase, and 0.1 U/ml catalase). The final concentrations were 20 nM Myo4p–She3p and 5 nM She2p–YFP. Nethylmaleimide–modified skeletal muscle myosin forms a strong and ATP-insensitive bond with actin and was used to attach the actin filaments to the coverslip.

Online supplemental material

Fig. S1 shows gels of expressed Myo4p–She3p and a FLAG resin pull-down assay. Fig. S2 compares the fluorescence intensity of She2p–YFP and myosin Vo–YFP. Fig. S3 shows the stepping pattern of Myo4p–She3p bound to She2p. Video 1 shows a Myo4p–She3p–She2p motor complex moving processively on an actin filament. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201106146/DC1.

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References


Supplemental material

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Figure S1. **Gels of expressed Myo4p–She3p and a FLAG resin pull-down assay.** (A) SDS gel of baculovirus-expressed and purified Myo4p–She3p expressed with yeast CaM alone (lane 1) or both yeast CaM and Mic1p (lane 2). Mic1p was His tagged, and CaM was Myc tagged (see Materials and methods). The protein used in this study was expressed with both Mic1p and yeast CaM. Both proteins bind to the lever arm, with Mic1p as the predominant light chain. Molecular masses of the proteins are indicated. HC, heavy chain. (B) A FLAG-tagged truncated Myo4p–She3p construct lacking the head was incubated with either WT* or the Δhelix-She2p construct. FLAG resin was used to isolate the complex, which was analyzed by SDS gels. Deletion of the helix abolished the ability of She2p to bind the Myo4p–She3p complex. Molecular masses of the proteins are indicated.

Figure S2. **Comparison of fluorescence intensity of She2p-YFP versus myosin Va-YFP.** The intensity of tetrameric She2p-YFP was compared with that of myosin Va (MyoVa–YFP). Myosin Va is a known dimer and therefore contains exactly two YFP molecules. Myosin Va–YFP and She2p-YFP were adsorbed directly to a glass coverslip and imaged by TIRF microscopy. The histogram shows a comparison of intensities (peak intensity with background subtracted) for myosin Va–YFP (n = 189) and She2p-YFP (n = 169). As expected, the intensity distribution for each followed a log-normal distribution, shown as solid lines (Mutch et al., 2007). The distribution for She2p-YFP shifted right toward higher intensities, relative to myosin Va–YFP. The peak intensity of She2p-YFP was 63.5 ± 1.7 versus a peak intensity of 40.4 ± 1.3 for myosin Va, corresponding to ~3.1 YFPs per She2p. This result indicates that we are visualizing single She2p tetramers and not larger aggregates. Arb. Unit, arbitrary unit.
Figure S3. **Stepping pattern of Myo4p–She3p bound to She2p.** A single head of a Myo4p–She3p complex bound to She2p was labeled with a Qdot. Qdot displacement versus time is shown by open circles. Qdot positions were determined by the ImageJ plugin SpotTracker (Sage et al., 2005). Steps were identified using the Kerssemakers step-finding algorithm (solid lines; Kerssemakers et al., 2006). Each plot shows a single representative trace. A total of 15 traces from three experiments was analyzed.

Video 1. **A Myo4p–She3p–She2p motor complex moving processively on an actin filament.** Myo4p–She3p was mixed with She2p-YFP and visualized by TIRF microscopy. YFP particles (green) were observed to move processively along Alexa Fluor 594-labeled actin filaments (red) in 1 mM MgATP. The field of view is 5.3 × 7.4 µm. Data were collected at 10 frames per second and played back at 30 frames per second.

**References**