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Engineering myosins for long-range transport on actin filaments

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Abstract

Cytoskeletal motors act as cargo transporters in cells¹ and may be harnessed for directed transport applications in molecular detection and diagnostic devices². High processivity — the ability to take many steps along a track before dissociating³ — is often a desirable characteristic because it allows nanoscale motors to transport cargoes over distances of microns *in vivo* and *in vitro*. Natural processive myosins^{4,5} are dimeric and use internal tension to coordinate the detachment cycles of the two heads^{6–8}. Here, we show that processivity can be enhanced in engineered myosins using two non-natural strategies designed to optimize the effectiveness of random, uncoordinated stepping: (i) formation of three-headed and four-headed myosins; and (ii) introduction of flexible elements between heads. We quantify improvements using systematic single-molecule characterization of a panel of engineered motors. To test the modularity of our approach, we design a controllably bidirectional myosin that is robustly processive in both the forward and backward direction, and also produce the fastest processive cytoskeletal motor measured to date, reaching a speed of 10 $\mu\text{m/s}$.

Previous investigations have shown that processive myosin motion can be tolerant to dramatic changes in lever arm structure and mechanics^{9,10}. These studies challenged the idea that processivity depends critically on head-head coordination mediated by internal strain: while natural dimeric motors such as myosin V and myosin VI make use of strain-dependent gating of nucleotide exchange to prevent unproductive detachment of the front head^{6–8}, this mechanism may yield only modest gains in processivity relative to

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Contributions: T.D.S. and Z.B. designed the myosin constructs. T.D.S. and L.C. performed experiments on the bidirectional design target. T.D.S. and P.L. performed gold nanoparticle tracking experiments. T.D.S. performed all other experiments with assistance from M.N. and L.C., and analysed the experimental data. Z.B. conceived and supervised the project. T.D.S. and Z.B. wrote the paper. All authors discussed the results and commented on the manuscript.

Website Summary: Diverse myosins can be modified by non-natural means to obtain high processivity on actin filaments.

uncoordinated stepping. Processivity without coordination has also been observed and discussed in recent studies of cytoplasmic dynein^{11,12}. Processive motion of a dimeric motor can be explained by the statistics of independently cycling monomers, dependent on kinetic properties of the catalytic head: specifically, the motor variant must have a high *duty ratio*, defined as the fraction of the mechanochemical cycle spent bound to the filament³.

Models of uncoordinated stepping suggest that trimeric or tetrameric myosins — which have not been observed in nature — could be dramatically more processive than dimers. Potential gains are illustrated by a toy kinetic model that has been useful for conceptual discussions of processivity^{7,10}, in which the mechanochemical pathway of a myosin head is approximated by a two-state cycle governed by an attachment rate k_{on} and a detachment rate k_{off} . With three or four heads and a high duty ratio $r=k_{on}/(k_{on}+k_{off})$, the termination of a run by simultaneous detachment of all heads becomes an unlikely event (Supplementary Modeling). This simple picture ignores potential detrimental effects of additional heads, which could include disruption of natural strain-mediated nucleotide gating, unintentional introduction of unproductive gating, or interference with actin rebinding due to mechanical or steric interactions between heads or due to site occlusion.

To test whether increasing the number of heads can improve the processivity of engineered motors, we designed a series of constructs based on myosin VI. Myosin VI has a high duty ratio⁵ of 0.85–0.95^{10,13} and is processive in dimeric constructs^{5,10}, including chimeric dimers with artificial lever arms composed of 2 spectrin repeats^{9,10}. We created M6DI₈₁₆2R~DIM, M6DI₈₁₆2R~TRI, and M6DI₈₁₆2R~TET by fusing the chimeric myosin VI motor to engineered coiled coil variants¹⁴ GCN4-pIL, GCN4-pII, and GCN4-pLI (DIM, TRI, and TET) to generate dimeric, trimeric, and tetrameric motors, respectively (Fig. 1) (Supplementary Figure 5). We used single-fluorophore tracking (Supplementary Movies 1–2) to quantify the processivities of the engineered constructs. For all ATP concentrations investigated, motor trajectories (Fig. 1a) show run lengths that increase steeply with increasing oligomerization state (Fig. 1b). M6DI₈₁₆2R~TRI and M6DI₈₁₆2R~TET suffer only minor reductions in velocity relative to their dimeric counterpart (Fig. 1c).

To further characterize the behaviour of the M6DI₈₁₆2R constructs, we analysed motor trajectories at high resolution using evanescent darkfield gold nanoparticle tracking¹⁵. Individual steps were observable in M6DI₈₁₆2R~DIM, TRI, and TET trajectories (Fig. 1d). Step size distributions depend on the oligomerization state (Fig. 1e); trimers and tetramers show increased frequencies of backsteps and short steps relative to dimers. Overall, trimers and tetramers have slightly shorter mean displacements per step, but many more steps per diffusive encounter.

Shortening the lever arms of our dimers, trimers, and tetramers results in substantially reduced run lengths. M6DI₈₁₆1R~DIM, M6DI₈₁₆1R~TRI, and M6DI₈₁₆1R~TET (Fig. 2a) were generated by truncating the lever arms of the M6DI₈₁₆2R constructs to only one spectrin repeat (1R). Only M6DI₈₁₆1R~TET yields measurably processive runs, and these runs are observable only at low [ATP] (Fig. 2a, b). Engineered myosins with shortened lever arms have displayed low processivities in a number of previous studies^{16,17}, including our work on controllable bidirectional myosins¹⁸. Possible causes include inhibition of

rebinding due to increased strain in 2-head-bound states^{16,17,19}, aberrant gating due to high and/or off-axis strain, and inaccessibility of preferred off-axis sites due to surface-bound filaments.

To address possible causes of reduced processivity, we created modified M6DI₈₁₆1R~ constructs in which we added a “slack” element consisting of a spectrin repeat flanked with flexible (Gly-Ser-Gly)₄ hinges on both sides (Fig. 2). Without extending the rigid lever arm, this slack element is expected to provide increased conformational freedom, reduce internal strain, and provide easier access to potential binding sites. The additional compliance should abrogate strain-mediated gating, which could reduce processivity if gating is beneficial but may increase processivity if unproductive gating is present. The addition of slack results in dramatic run length increases relative to the original M6DI₈₁₆1R constructs (Fig. 2, Table 1). M6DI₈₁₆1R~1R~TET exhibits a mean run length of $\langle L \rangle = 6.87 \pm 0.80 \mu\text{m}$ (mean $\pm$ s.e.m.) at 2 mM ATP. In comparison, M6DI₈₁₆1R~TET is not processive at 2 mM ATP, and has a mean run length of only $\langle L \rangle = 0.24 \pm 0.02 \mu\text{m}$ at 50 μM ATP.

We created additional constructs to verify that the beneficial effect of additional compliance does not depend on having more total spectrin repeats in the motor. M6DI₈₁₆~1R~TRI and M6DI₈₁₆~1R~TET (Fig. 2, Table 1) differ from M6DI₈₁₆1R~TRI and M6DI₈₁₆1R~TET only via the insertion of a (Gly-Ser-Gly)₄ hinge (~) between the motor domain and lever arm. Both constructs have much longer mean run lengths than their M6DI₈₁₆1R counterparts (Fig. 2, Table 1) (Supplementary Movie 3).

We tested the generalizability of our multimerization and slack strategies by constructing engineered motors based on myosin XI, a class with properties that differ significantly from myosin VI. Myosins in this fast and plus-end directed class^{20,21} are found in plants and algae and are responsible for transport of golgi stacks, peroxisomes, and mitochondria²², and for cytoplasmic streaming²³. Processive motion of wild-type myosin XI dimers purified from *Nicotiana tabacum* has been recorded²¹ at 7 $\mu\text{m/s}$, the fastest previously observed processive movement of a cytoskeletal motor. No processive behaviour has been reported for other native myosin XI complexes (such as the well-studied *Chara corallina* motor, which is reported to have a low duty ratio of ~0.34–0.49²⁰) or for any recombinantly produced myosin XI protein.

We created engineered myosin XI motors that lack any rigidly attached lever arms, fusing only our slack element ~1R~ and engineered coiled coil domains (DIM, TRI, TET) to the catalytic domain (CD) of myosin XI from *Nicotiana tabacum* (NM11) or *Chara corallina* (CM11). We measured processive motion for NM11CD₇₃₈~1R~DIM, TRI, and TET, and CM11CD₇₄₆~1R~DIM and TET (Fig. 3a). As seen with our myosin VI constructs, processivity increased with oligomerization state (Fig. 3b). These motors move substantially faster than the myosin VI multimers (Fig. 3c) (Supplementary Movie 4).

Unexpectedly, a comparison of the closely related CM11CD₇₄₆~1R~ and NM11CD₇₃₈~1R~ constructs also illuminates specialized properties of the *Chara* motor. At most ATP concentrations, CM11CD₇₄₆~1R~ constructs are both faster and more processive than their NM11CD₇₃₈~1R~ counterparts. When comparing myosin classes, there is often a trade-off

between processivity and speed²⁴, which may be understood on the basis of simple kinetic competition (Supplementary Modeling): a short-lived actin-bound state favours high velocities, but (because detachment of the bound head competes with attachment of the free head) disfavors processivity. The simultaneous advantage in speed and processivity of *Chara* M11 over *Nicotiana* M11 may be related to the unusual charge distribution in *Chara* myosin actin binding loops²⁵, which has previously been proposed to enable high speed without sacrificing actin affinity.

Having developed a general approach for engineering processivity, we applied it to the design of two processive motors with two different properties desirable for potential applications: (i) controllable bidirectionality and (ii) high speed. For both design targets, we chose to make tetramers with slack elements to achieve maximum processivity while counteracting potential detrimental effects from short lever arm lengths.

We previously engineered a chemically controlled bidirectional myosin that switches direction in response to calcium concentration¹⁸. M_{CaR}-2IQ-DIM exhibits processive motion in both (+) and (−) directions. However, this construct is not measurably processive at saturating ATP, and shows only moderate run lengths even at 50 μM ATP: $\langle L \rangle = 550$ nm for (−)-end directed motion and $\langle L \rangle = 170$ nm for (+)-end directed motion. Here, we created M_{CaR}-2IQ~1R~TET (Fig. 4a) by fusing M_{CaR}-2IQ to a flexible (Gly-Ser-Gly)₄ hinge, a spectrin repeat (1R), an additional hinge, and the tetrameric GCN4-pLI coiled coil (TET). M_{CaR}-2IQ~1R~TET is highly processive and has mean run lengths at 2mM ATP that exceed those of M_{CaR}-2IQ-DIM at 50 μM ATP (Fig. 4b). High processivity facilitates new experiments in which individual motors are dynamically switchable via *in situ* buffer exchange (Fig. 4c) (Supplementary Movies 5A–C).

Chara corallina myosin XI is the fastest known cytoskeletal motor²⁶. Our results (Fig. 3) showed that *Chara* myosin can be incorporated into processive tetramers, although no processive behaviour has previously been reported. We reasoned that if we could increase the speed of our processive *Chara* constructs by adding a rigidly attached lever arm, we might achieve velocities exceeding any known processive cytoskeletal motors. Building on previous work in which the lever arm of the *Chara* myosin was successfully replaced with two spectrin repeats (2R)²⁷, we generated a tetrameric construct containing a 2R lever arm and a ~1R~ slack element. CM11CD₇₄₆2R~1R~TET (Fig. 4d) remains measurably processive at velocities up to $\langle V \rangle = 10$ μm/s (Fig. 4e) (Supplementary Movie 6). To our knowledge, this is the fastest processive speed measured for a cytoskeletal motor.

Previous work on multimotor cargo transport has established that dimeric processive motors have longer run lengths when assembled into teams of two or more dimers^{28–32}. Using bead assemblies^{29,33} or DNA scaffolds^{28,30,31}, this effect has been observed for kinesin-1^{28,29,31,32}, myosin V³⁰, and dynein^{28,33}. Here, we have shown that engineered monomers can be assembled into processive complexes; fine-tuned coordination and native dimer architectures are disposable^{9,10}, and effective processivity enhancement can be achieved by forming multiheaded constructs and introducing flexible spacers designed to increase diffusive freedom while abrogating internal tension. Quantitative improvements in processivity, while smaller than seen for a toy model that ignores interference effects and

other details (Supplementary Figure 3), are larger than has generally been reported for comparable multimotor assemblies: for both M6DI₈₁₆2R and CM11CD₇₄₆ constructs, the tetramer is ~10X more processive than the dimer, which may be compared with a ~2X improvement in processivity for coupling two myosin V dimers together using a DNA scaffold³⁰. Our measurements complement the recent discovery of processive motion in small defined assemblies of kinesin-14 dimers, which are non-processive in isolation³².

This work provides a general approach for increasing the processivities of engineered myosins in synthetic biology or nanotechnology applications. The modularity of the approach allows high processivity to be combined with complex engineered properties such as directionality control, or with higher velocities than are normally associated with processive motors. Engineered complexes are simply and efficiently assembled (Supplementary Figure 5) upon expression of a single polypeptide chain, and should be deployable *in vivo* for modifying the parameters of cargo transport processes³⁴. Engineered motors may also be used for *in vitro* imitations of these cellular systems, which achieve effective long-range transport through a combination of processive stepping, high filament densities (to favour rebinding after dissociation), and high motor velocities³⁵. In diagnostic device designs that use molecular motors to overcome diffusion limits for sensitive detection³⁶ or perform molecular sorting tasks³⁷, long-range transport has often been implemented using filament-based cargo carriers². New processive motors will enhance the capabilities of alternative designs using motor-based cargo carriers³⁸, allowing for further miniaturization and molecular customization and control.

METHODS

Proteins

DNA constructs were assembled from modules encoding porcine myosin VI (residues 1–816), *Chara corallina* myosin XI (residues 1–746), *Nicotiana tabacum* myosin XI (residues 1–738), *Dictyostelium* α -actinin (residues 266–388 for 1R and residues 266–502 for 2R), and engineered GCN4 leucine zipper variants pIL, pII, and pLI¹⁴ (Supplementary Figure 4). All constructs contained a C-terminal flexible linker, HaloTag protein sequence (Promega), and flag tag (DYKDDDDK) and were cloned into pBiEx-1 (Novagen). Proteins were expressed via direct transfection of SF9 cells and purified and labeled with TMR HaloTag ligand (Promega) or PEG-biotin HaloTag ligand (Promega) as previously described^{9,10}.

Single-molecule fluorescence tracking assays

Assays were carried out in Assay Buffer (AB) containing 25 mM imidazole, 25 mM KCl, 1 mM EGTA, and MgCl₂. The concentration of MgCl₂ added varied depending on final assay ATP concentration to give 2mM free Mg²⁺, according to MAXCHELATOR (<http://maxchelator.stanford.edu>). Calmodulin (4 μ M) was added when assaying myosin VI constructs. Blocking buffer consisted of AB with 1 mg/mL BSA (Sigma). Imaging buffer consisted of AB with the desired ATP concentration, 1 mM phosphocreatine, 100 μ g/mL creatine phosphokinase, 0.4% glucose, 0.2mg/mL glucose oxidase, 36 μ g/mL catalase, and 1.8mM trolox. MCaR-2IQ~1R~TET constructs were assayed in buffers with 2 μ M calmodulin and either pCa 4 or EGTA as described¹⁸, with MgCl₂ added to yield 2 mM free

Mg²⁺ upon addition of 2 mM ATP in imaging buffer. Assays were performed essentially as described¹⁰, except that plasma-cleaned coverslips were used without any nitrocellulose coating. Briefly, *N*-ethyl maleimide inactivated full-length skeletal muscle myosin (a gift from R. Cooke) was used to immobilize actin filaments stabilized with excess Alexa 633 phalloidin (Invitrogen). Nonspecific binding was minimized by incubation with blocking buffer. Motors were added in imaging buffer. Videomicroscopy data were collected on an electron multiplying charge coupled device camera (Andor) under objective-side total internal reflection fluorescence excitation using a 532 nm diode-pumped solid-state laser (Coherent) through a 1.49 NA 100x objective (Nikon). Actin filaments were imaged prior to recording assay videos with a separate emission filter set. Motor trajectories were tracked using custom software as described¹⁰. To avoid artefacts due to undersampling of short runs¹⁰, an analysis cut-off was used: only trajectories lasting at least three frames and traveling more than two pixels (216 nm) were scored for velocity, run length, and directionality. For the high-velocity motor CM11CD₇₄₆2R~1R~TET assayed at 100 μ M and 500 μ M ATP, the cut-off was increased to three pixels (324 nm) (Supplementary Table 2). Mean run lengths were corrected for runs that terminated at the end of a filament using the Kaplan-Meier survivor function, and the reported errors of the mean (s.e.m.) are the error estimates from this function (Supplementary Figure 6). Constructs with mean run lengths $\langle L \rangle$ greater than two s.e.m. above one pixel (108 nm) were deemed “measurably processive”. “Mixed” runs for MCar-2IQ~1R~TET were defined as runs that showed >216 nm movement in one direction and subsequently >216 nm in the other direction. Velocity error estimates were calculated as described¹⁰, except that s.e.m. weighting was not used and the number of sets used in bootstrapping was equal to the number of runs scored. Fit error estimates for model fit parameters were calculated as described¹⁰. A summary of run length and velocity data can be found in Supplementary Table 1.

Gold nanoparticle tracking assays

NeutrAvidin-coated 50 nm gold nanospheres (Nanopartz) were washed into binding buffer (500 mM NaCl, 40 mM Tris pH 7.5, 0.2% (v/v) tween-20 (Sigma), and 1 mg/mL BSA) by centrifugation and resuspension. PEG-biotin labeled proteins were incubated with washed beads for 15 min at 25°C before dilution into imaging buffer. Assays were carried out as for single-molecule fluorescence tracking assays above with the addition of 0.2% (v/v) tween-20 in the blocking buffer and 1 mg/mL BSA and 0.2% tween-20 (v/v) in the imaging buffer, which contained 50 μ M ATP. Gold particles were imaged using objective-side evanescent darkfield microscopy. Laser illumination was provided by a 532 nm diode-pumped solid-state laser (Spectra-Physics) through a 1.49 NA 60x objective (Nikon) and totally internally reflected at the sample interface; additional details of the microscope design will be presented elsewhere (Lebel et al., submitted). Scattered light was imaged on a high-speed CMOS camera (Mikrotron Ecosens CL) at 500 Hz. Analysis details are provided in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Multimerization effects on engineered myosin VI processivity and stepping behaviour. **(a)** Single molecule fluorescence trajectories for M6DI₈₁₆2R dimers (red), trimers (green), and tetramers (blue) recorded at 2mM ATP together with corresponding cartoons and block diagrams, showing differences due to oligomerization state. **(b)** Mean run lengths as a function of [ATP]. M6DI₈₁₆2R~DIM is fit to a 3-state model¹⁰, yielding $k_{\text{rebind}} = 290 \pm 30 \text{ s}^{-1}$, $k_{\text{ADP off}} = 5.8 \pm 0.1 \text{ s}^{-1}$, $k_{\text{ATP on}} = 0.015 \pm 0.001 \text{ s}^{-1} \mu\text{M}^{-1}$, and the defect parameter $d = 63 \pm 6$ steps. **(c)** Motor velocities. M6DI₈₁₆2R~DIM is fit to the 3-state model, and M6DI₈₁₆2R~TRI and M6DI₈₁₆2R~TET are fit to Michaelis Menten kinetics. Trimer: $K_M = 740 \pm 40 \mu\text{M}$ and $V_{\text{max}} = 120 \pm 2 \text{ nm/s}$; tetramer: $K_M = 630 \pm 50 \mu\text{M}$ and $V_{\text{max}} = 99 \pm 3 \text{ nm s}^{-1}$. Data are displayed as mean $\pm$ s.e.m. **(d-e)** Gold nanoparticle tracking motor stepping traces. **(d)** Raw trajectories (colored traces, collected at 500 Hz) are shown along with fits (black) generated by an automatic stepfinding procedure (see Methods). **(e)** Step size distributions. Histograms of displacements generated by stepfinding ($N > 1000$ steps for each construct) are shown together with fits (black) to sums of multiple Gaussian distributions. Peak locations for dimer: $-12 \pm 0.1 \text{ nm}$ and $20 \pm 0.3 \text{ nm}$; trimer: $-9 \pm 0.1 \text{ nm}$, $9 \pm 0.1 \text{ nm}$, and $21 \pm 0.3 \text{ nm}$; tetramer: $-10 \pm 0.1 \text{ nm}$, $10 \pm 0.1 \text{ nm}$, and $23 \pm 0.3 \text{ nm}$. Mean displacements per step for dimer: $17.5 \pm 0.4 \text{ nm}$; trimer: $14.4 \pm 0.4 \text{ nm}$; tetramer: $13.2 \pm 0.5 \text{ nm}$.

Figure 2.

Effects of increased flexibility on myosin VI constructs with short lever arms. Single molecule fluorescence trajectories for dimers (red), trimers (green), and tetramers (blue) were recorded at 2mM ATP or (*) 50 μM, showing differences due to addition of a slack element (~1R~) or insertion of a flexible hinge (~).

Figure 3. Characterization of engineered myosin XI motors. **(a)** Cartoons and block diagrams for constructs using the *Nicotiana tabacum* (light brown) and *Chara corallina* myosin XI (dark brown) catalytic domains. **(b)** Run lengths and **(c)** velocities are shown (mean $\pm$ s.e.m.) as a function of [ATP].

Figure 4.

Characterization of design targets for combining processivity with other desirable characteristics. **(a)** MCaR-2IQ~1R~TET schematic. **(b)** MCaR-2IQ~1R~TET is processive at 2mM ATP in both low (EGTA) and high (pCa 4) calcium conditions. Randomly selected motor trajectories illustrate predominantly minus-end directed movement in EGTA (99.4% minus (dark blue), 0.6% plus, N=165) and predominantly plus-end directed movement in pCa 4 (83.3% plus (light blue), 2.1% mixed (gray), and 14.6% minus (dark blue), N=96). Minus-end directed run lengths in EGTA and plus-end directed run lengths in pCa 4 are displayed as mean $\pm$ s.e.m. Filament orientations were determined using M6DI₈₁₆2R~DIM as a minus-end directed control¹⁸. Average velocities were $\langle V \rangle = 8 \pm 1$ nm/s for minus-end directed runs in EGTA and $\langle V \rangle = 1 \pm 0.1$ nm/s for plus-end directed runs in pCa 4 (mean $\pm$ s.e.m.). **(c)** Dynamic switching of individual motors was accomplished using *in situ* buffer exchange. Duration of buffer exchange is approximate. **(d)** CM11CD₇₄₆2R~1R~TET schematic. **(e)** CM11CD₇₄₆2R~1R~TET is processive over a range of [ATP], processive velocities up to $\langle V \rangle = 10.0 \pm 0.3 \mu\text{m s}^{-1}$. Velocity and run length data are shown as mean $\pm$ s.e.m.

Table 1

Effects of increased flexibility on myosin VI constructs with short lever arms.

Construct	Run Length, $\langle L \rangle$ (μm)
M6DI ₈₁₆ 1R~DIM	not measurable (*)
M6DI ₈₁₆ 1R~1R~DIM	0.64 ± 0.05 (*)
M6DI ₈₁₆ 1R~TRI	not measurable
M6DI ₈₁₆ 1R~1R~TRI	2.20 ± 0.14
M6DI ₈₁₆ 1R~TET	not measurable
M6DI ₈₁₆ 1R~1R~TET	6.87 ± 0.80
M6DI ₈₁₆ 1R~TRI	not measurable
M6DI ₈₁₆ ~1R~TRI	0.44 ± 0.05
M6DI ₈₁₆ 1R~TET	0.24 ± 0.02 (*)
M6DI ₈₁₆ ~1R~TET	7.66 ± 0.35 (*)

Mean run lengths are compared between 1R and 1R~1R (top) and between 1R and ~1R (bottom) for single molecule trajectories (Fig. 2) acquired at 2mM or (*) 50 μM ATP. Data are shown as mean $\pm$ s.e.m.