The Light Chain Binding Domain of Expressed Smooth Muscle Heavy Meromyosin Acts as a Mechanical Lever*

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Structural data led to the proposal that the molecular motor myosin moves actin by a swinging of the light chain binding domain, or “neck.” To test the hypothesis that the neck functions as a mechanical lever, smooth muscle heavy meromyosin (HMM) mutants were expressed with shorter or longer necks by either deleting or adding light chain binding sites. The mutant HMMs were characterized kinetically and mechanically, with emphasis on measurements of unitary displacements and forces in the laser trap assay. Two shorter necked constructs had smaller unitary step sizes and moved actin more slowly than WT HMM in the motility assay. A longer necked construct that contained an additional essential light chain binding site exhibited a 1.4-fold increase in the unitary step size compared with its control. Kinetic changes were also observed with several of the constructs. The mutant lacking a neck produced force at a somewhat reduced level, while the force exerted by the giraffe construct was higher than control. The single molecule displacement and force data support the hypothesis that the neck functions as a rigid lever, with the fulcrum for movement and force located at a point within the motor domain.

Muscle contracts as a result of the cyclic interaction of the molecular motor myosin with actin, powered by the hydrolysis of MgATP. A simple mechanistic model by which myosin could move actin was proposed based on the crystal structure of skeletal myosin subfragment 1 (1–3). The key feature was an 8.5-nm single a-helix, stabilized by the essential and regulatory light chains (ELC and RLC), which formed an elongated neck region that emerged from the globular motor domain. It was suggested that a substantial portion of the myosin motor domain maintains a fixed orientation when attached to actin, while the neck region pivots about a fulcrum within the motor domain, thus generating a power stroke.

Additional evidence in support of a lever arm rotation was obtained from the crystal structure of a motor domain-essential light chain complex with a transition state analog at the active site, which showed the lever arm in a second position that may represent myosin in the prepowerstroke state (4). The skeletal subfragment 1 structure is likely to resemble the structure adopted at the end of the powerstroke. A comparison of the two conformations shows that smaller changes that originate at the active site are amplified into much larger movements of the lever arm. This motion could accommodate a powerstroke on the order of 10 nm, in the range of the 5–15 nm of displacement measured using single molecule techniques (5–8).

The simplest mechanical model for the neck region predicts that myosin with a shorter neck (i.e. shorter lever arm) should generate smaller unitary displacements and move actin more slowly, whereas a longer neck should lead to larger displacements and more rapid actin movement (reviewed in Ref. 9). Several studies in which myosins of various neck lengths were produced either by removing light chains (10, 11) or by genetically adding or deleting light chain binding sites (12, 13) showed that constructs with necks shorter than wild type moved actin more slowly, while a construct with a longer neck moved actin more quickly. A chimera in which two a-actinin repeats were fused to the Dictyostelium motor domain showed a higher average velocity than a similar construct with only one a-actinin repeat, suggesting that nonnative structures can mimic some aspects of the native neck (14). Although these studies are consistent with a simple lever arm model, they all relied on the assumption that no kinetic changes resulted from these biochemical or genetic perturbations. Since velocity in the motility assay, \( v_{max} \), is dependent upon both step size \( \Delta d \) and the time spent attached to actin (15), changes in either parameter could equally well account for the observed differences in motility.

Here we test the lever arm hypothesis at the single molecule level by measuring the displacement \( \Delta d \) and force \( F \) of a series of smooth muscle heavy meromyosin (HMM) mutants in which light chain binding sites were either added to or deleted from the neck. The laser trap data support the hypothesis that the neck acts as a lever and are consistent with structural data that suggest that the fulcrum for movement and force is located near the SH1 helix (4).

**EXPERIMENTAL PROCEDURES**

Protein Preparation and Expression—Wild type (WT) smooth muscle HMM (amino acids 1–1175) and neck length mutants of this heavy chain backbone were co-expressed with the regulatory and essential light chains using the baculovirus insect cell expression system (11). The HMM backbone was chosen as the basis for the different neck-length constructs so that monoclonal anti-rod antibody S2.2 could be used as a common means of attaching the molecules to the nitrocellu-
lose substrate for both motility and laser trap studies. Proteins were purified by binding to actin and release with MgATP as described previously (11).

Design of Mutants—Two constructs with neck lengths shorter than WT HMM were cloned (see Fig. 1). An HMM with no light chain binding region (“neckless”) had amino acids 820–849 deleted. This resulted in a dimeric construct with the motor domain attached to the rod. The sequence of the region joining the motor domain to the rod in the neckless construct was ERDLGPLLQV. An HMM mutant lacking an RLC binding site (“-R site”) had amino acids 820–849 deleted. The sequence of the joining region in this construct, which contains the motor domain, an ELC binding site attached to the rod, was QQQLGLPQQV.

A long necked mutant (“giraffe”) in which a second ELC binding site was added between the native ELC and RLC binding sites was also cloned (Fig. 1). This mutant retains native contacts between the motor domain and the ELC and between the ELC and the RLC but introduces a foreign ELC–ELC interaction. The following sequence was introduced between Leu<sup>819</sup> and Thr<sup>820</sup>: LIGTVIIFQQACRGYLRKAFKAKRQQQL. This sequence is LG plus amino acids 792–819. To test if the orientation of the two ELCs with respect to each other influences the properties of the giraffe construct, a second variation was constructed (“giraffe’s twisted sister”). This revised construct had one less amino acid in the added ELC site than the previous construct; thus, the two ELCs were rotated by approximately 100°. This was accomplished by inserting the sequence of amino acids 791–819 (i.e. RTTDV1-IAPFAQCQCRGLYKFAKARQQQQL) between Leu<sup>819</sup> and Thr<sup>820</sup>. A phosphorylation-independent variant of giraffe HMM was also engineered. Based on earlier studies, substitution of the 50/20-kDa β-cardiac actin binding loop (CABL) for the native sequence (residues 626–653) activated unphosphorylated smooth muscle HMM (16). Thus, the double mutants, CABL-giraffe and CABL-giraffe’s twisted sister, were also constructed. The movement of these constructs was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed.

Unphosphorylated CABL-HMM with a native neck was used as the control for the unphosphorylated molecule studies could be more readily performed. Unphosphorylated CABL-giraffe was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed. A phosphorylation-independent variant of CABL-giraffe was also engineered. Based on earlier studies, substitution of the 50/20-kDa β-cardiac actin binding loop (CABL) for the native sequence (residues 626–653) activated unphosphorylated smooth muscle HMM (16). Thus, the double mutants, CABL-giraffe and CABL-giraffe’s twisted sister, were also constructed. The movement of these constructs was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed. Unphosphorylated CABL-HMM with a native neck was used as the control for the unphosphorylated CABL-giraffe.

Biochemical Characterization of Expressed Constructs—Actin-activated ATPase assays were performed in 10 mM imidazole, pH 7, 8 mM KC1, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaN<sub>3</sub> at 37 °C. Inorganic phosphate was determined colorimetrically at six time points per actin concentration, using SDS to stop the reaction (17). The concentration of active heads was determined by NH<sub>4</sub>Cl–ATPase activity relative to a myosin standard (25 mM Tris, pH 7.5, 37 °C, 0.4 mM NH<sub>4</sub>Cl, 0.2 mM EGTA, 0.2 mM sucrose, 1 mM dithiothreitol, 1 mM Mg<sub>2</sub>Cl<sub>2</sub> bovine serum albumin). A phosphorylation-independent variant of CABL-giraffe was also engineered. Based on earlier studies, substitution of the 50/20-kDa β-cardiac actin binding loop (CABL) for the native sequence (residues 626–653) activated unphosphorylated smooth muscle HMM (16). Thus, the double mutants, CABL-giraffe and CABL-giraffe’s twisted sister, were also constructed. The movement of these constructs was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed. Unphosphorylated CABL-HMM with a native neck was used as the control for the unphosphorylated CABL-giraffe.

Electron Microscopy—Rotary-shadowed platinum images were obtained in 0.5 m ammonium acetate, 66% glycerol and observed with a Philips EM301 electron microscope operated at 60 kV (18). Actin was decorated with the expressed constructs, negatively stained, and observed over holes on carbon-coated grids (19).

In the motility assay, which was performed at 30 °C in 25 mM imidazole, pH 7.5, 25 mM KC1, 4 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% methacrylate, as described by Trybus and Chatman (20), CARL-phorylated long necked CABL-giraffe. CABL-HMM with a native neck was used as the control for the unphosphorylated molecule studies could be more readily performed. Unphosphorylated CABL-giraffe was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed. A phosphorylation-independent variant of CABL-giraffe was also engineered. Based on earlier studies, substitution of the 50/20-kDa β-cardiac actin binding loop (CABL) for the native sequence (residues 626–653) activated unphosphorylated smooth muscle HMM (16). Thus, the double mutants, CABL-giraffe and CABL-giraffe’s twisted sister, were also constructed. The movement of these constructs was more consistent than the original giraffe construct, and thus the single-molecule studies could be more readily performed. Unphosphorylated CABL-HMM with a native neck was used as the control for the unphosphorylated CABL-giraffe.

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RESULTS

Characterization of Shorter Necked Constructs—Two double-headed constructs with neck lengths shorter than that of wild type HMM were engineered and expressed in the baculovirus/insect cell system (Fig. 1). One construct lacked an RLC binding site (“-R site”), and SDS-gels confirmed that this construct did not bind RLC (Fig. 2C). The second shorter necked construct lacked both an RLC and an ELC binding site (neckless). Metal-shadowed images showed that the two globular domains closely abut the rod, as would be expected from a neckless construct that lacked the light chain binding domain (Fig. 2A). These characterizations, as well as the functional assays that follow, are necessary to ensure that the constructs that are analyzed in the laser trap retain certain essential and characteristic features of myosin.

To assess if the mutations altered the kinetics of the cross-bridge cycle, actin-activated ATPase measurements were performed. Since both shorter necked constructs lacked the RLC, these molecules did not require phosphorylation for activation. The unactivated ATPase activity of -R site (open squares) and neckless (filled triangles) was similar to that of phosphorylated WT HMM (filled circles) (Fig. 3A).

The mutants were also tested by an in vitro motility assay, which serves as a simplified model system to assess the motion-generating capacity of myosin at the molecular level. Both shorter necked constructs moved actin more slowly than WT before surface blocking with bovine serum albumin (500 μg/ml). HMM was then applied in concentrations varying from 2.5–10 μg/ml and allowed to bind to the antibody for 2 min. Thus, HMM was bound to the surface with random orientation but at a defined point on the molecule. All experiments were performed at low ionic strength (25 mM RCl), limiting ATP concentration (10 μM), and 25 °C, to prolong the unitary event durations.

To measure the displacements imparted to the actin filament by a single HMM molecule, the bright field image of one of the microsphere handles is projected onto a quadrant photodiode detector, providing x and y bead position data. Myosin’s unitary displacements were recorded under “unloaded” conditions (0.02–0.04 piconewton/nm/trap). To measure force, a nearly isometric condition was obtained under feedback control by increasing the effective trap stiffness approximately 100-fold. The feedback loop contained an acousto-optic modulator, which was used to deflect the laser trap and thus counterbalance the myosin-generated forces (7). The acousto-optic modulator control signal was calibrated and served as the force signal. Force measurements in this assay may not represent the true maximum unitary force due to compliance in the actin filament-bead attachment and the low bandwidth of the feedback system (i.e. ~140 Hz). Although the forces measured are surely underestimates, they are still meaningful for comparative purposes across mutants.

The technique of “mean-variance” (MV) analysis (25) was used to derive estimates of force and displacement from the laser trap data. MV analysis begins with a model-independent transformation of the time record, thus giving an alternative view of the data (a MV histogram), which emphasizes intervals of constant properties within the data. Generation of the MV histogram requires no assumptions about or interpretation of the underlying data, and quantitative descriptions of the data are derived from curve fits to the histogram. Thus, MV analysis is less prone to the biases introduced by manual scoring methods and may be used to estimate the size (i.e. d and F), distribution, number, and duration of events (t<sub>m</sub>) in the data as described previously (7, 24).

Neck Length Mutants in Smooth Muscle HMM

![Fig. 1. Schematic diagram of the constructs used in this study.](Image 345x651 to 517x729)
Neck Length Mutants in Smooth Muscle HMM

FIG. 2. Characterization of expressed myosin mutants. A, metal-shadowed images of neckless, giraffe, and WT HMM. In some of the images, it can be clearly seen that neckless has a shorter neck than WT and that giraffe has a longer neck than WT. Magnification was × 110,000. B, negative stained images of actin decorated with the long necked giraffe construct. The typical arrowhead appearance indicates regular binding despite the mutation of the neck region. Magnification was × 150,000. C, SDS-gel of tissue purified myosin (lane 1), and the mutant construct lacking the RLC binding site (-R site) (lane 2).

FIG. 3. Actin-activated ATPase activity of expressed constructs. A, rate of ATP hydrolysis as a function of actin concentration for phosphorylated WT HMM (●), dephosphorylated WT HMM (○), -R site (□), and neckless (▲). The solid line through the points is a fit with a $V_{\text{max}} = 4.6 \pm 1.7$ s$^{-1}$, but this value is approximate, since the $K_m$ values were −75 μM. B, rate of ATP hydrolysis as a function of actin concentration for dephosphorylated CABL-HMM (●) and dephosphorylated CABL-giraffe HMM (▲). The fits to the curves are $V_{\text{max}} = 2.9 \pm 0.3$ s$^{-1}$, $K_m = 16 \pm 5$ μM for CABL-HMM and $V_{\text{max}} = 6.7 \pm 0.6$ s$^{-1}$, $K_m = 6 \pm 5$ μM for CABL-giraffe HMM.

HMM. The neckless construct moved actin at ~25% of the velocity (0.26 ± 0.04 μm/s) of phosphorylated WT HMM (1.1 ± 0.19 μm/s), while -R site moved actin at ~45% the rate (0.49 ± 0.18 μm/s) of phosphorylated WT HMM (Fig. 2A). Analyses of mean displacement event durations, $t_{\text{on}}$, showed that the -R site mutant had shorter event durations than WT HMM (Table I).

Unitary forces were also recorded (Fig. 5, Table I). The appearance of force events within the time series data was similar to that of displacement events and thus analyzed by MV analysis. The neckless construct generated forces that were somewhat lower than that of WT HMM (~1.1 versus ~1.6 piconewtons, respectively).

Characterization of Longer Necked Constructs—If the lever arm model appropriately describes the mechanical properties of the neck region, then a longer neck should result in larger unitary displacements. Therefore, a longer necked “giraffe” construct with an additional ELC binding site was expressed. This type of construct is a more stringent test of the lever arm hypothesis (Fig. 1), given that a gain rather than a loss in function is expected.

Visual inspection of giraffe HMM by metal-shadowing showed that the construct appeared normal except for having an apparently longer neck region (Fig. 2A). In addition, the construct decorated actin in a regular manner, suggesting that its actin-binding properties were intact (Fig. 2B). The ratio of ELC/RLC was 1.7–1.9 times larger in the giraffe construct compared with WT HMM (average slope from four gel loadings, two independent preparations), consistent within experimental error with the construct containing an additional ELC binding site. However, despite having actin-activated ATPase activity, less than half of the actin filaments moved in the motility assay, and they moved at a rate at least 2-fold slower than phosphorylated WT HMM. The rates of motility were 0.49 ± 0.13 μm/s for phosphorylated giraffe HMM and 0.23 ± 0.04 μm/s for unphosphorylated giraffe HMM. The addition of exogenous ELC to the preparation had no effect on motility. When three independent preparations of the phosphorylated giraffe HMM were used in an attempt to obtain unitary displacement data, the data were of insufficient quality to perform the MV analysis.

Given that smooth muscle myosins’ ability to move actin is strictly dependent on light chain phosphorylation, we hypothesized that light chain phosphorylation was not able to fully activate the giraffe HMM construct. If this is true, then a constitutively active mutant that contains the same elongated neck region as the “wild type” giraffe construct should circumvent the problem of poor motility. We had previously shown that mutation of the actin-binding loop to the sequence found in cardiac myosin produced a constitutively active molecule (16),
Neck Length Mutants in Smooth Muscle HMM

Fig. 4. Motility of expressed neck-length mutants. A, velocity of movement (mean and S.D.) for the two shorter neck-length constructs compared with WT HMM. All three constructs were attached to the nitrocellulose substratum via monoclonal antibody S2.2. B, velocity of movement (mean and S.D.) of the longer necked giraffe construct (striped bars) compared with its WT control (solid bars). Four independent paired preparations of CABL-HMM and CABL-giraffe are shown. C, histogram of filament velocities (preparation 1, B). Velocities during 1-s intervals for at least 20 filaments are plotted. 213 1-s intervals were used for CABL-HMM, and 194 1-s intervals were used for CABL-giraffe.

Fig. 5. Laser trap time series data of single HMM molecule displacements and forces. Shown are 5-s records of displacement and force from WT HMM, a neckless mutant, CABL-WT (which is the control for a long necked mutant), and CABL-giraffe (see “Experimental Procedures” for construct details). For data analysis, records were typically 30–60 s long for a given HMM molecule and contained tens to hundreds of events per record. These raw data are shown for illustrative purposes. To estimate the displacement and force amplitudes from full-length records, mean-variance analysis was applied, and the results are reported in Table I. The noise is due to the Brownian motion of the bead-actin-bead assembly in solution. Individual events are seen as positive deflections with occasional negative deflections apparent (see force records for neckless and CABL-giraffe for examples).

and thus we expressed a long necked mutant, CABL-giraffe, that was active even when unphosphorylated (see “Experimental Procedures”). CABL-HMM with a native neck served as the control. Both constructs were analyzed in the unphosphorylated state. The actin-activated ATPase of CABL-giraffe (filled triangles) ($V_{max} = 6.7 \pm 0.6 \text{ s}^{-1}$, $K_m = 6 \pm 3 \text{ mM}$) was slightly more than twice than of CABL-HMM (filled circles) ($V_{max} = 2.9 \pm 0.3 \text{ s}^{-1}$, $K_m = 16 \pm 5 \text{ mM}$) (Fig. 3B). This result shows that the kinetics of the interaction with actin have been altered by the mutation in the neck.

When four independent preparations of CABL-giraffe were compared in parallel with CABL-HMM, there were no significant differences in motility, as would have been predicted from a simple lever arm model (Fig. 4B). One pair of preparations (Fig. 4B, I) was also analyzed by determining the velocities during 1-s intervals instead of obtaining an average velocity for a longer run, but still no significant differences emerged (Fig. 4C). This similarity in rates held true under standard motility conditions (25 mM KCl) as well as at higher salt concentrations (60 mM KCl). As previously reported, unphosphorylated CABL-HMM moved actin at ~50% the rate of phosphorylated WT HMM, and phosphorylation of CABL-HMM had only a slight effect, increasing this value to ~75% that of phosphorylated WT HMM (16).

A second long necked construct, CABL-giraffe’s twisted sister (see “Experimental Procedures”) was expressed in order to test if the relative orientation of the two ELCs had an impact on the rate of motility. The motility observed with this construct was $0.54 \pm 0.07 \text{ mm/s}$ (number of filaments = 26). This is within the range of values observed for both CABL-HMM and the original CABL-giraffe construct ($0.36–0.55 \text{ mm/s}$; see Fig. 4).

Fig. 6. Unitary step size is a linear function of relative lever arm length. Unitary displacements ($d$) for neck length mutants are plotted as mean ± S.D. against relative lever arm length ($L$). WT HMM or CABL-HMM, which contain two light chains, were assigned a relative lever arm length of 1. Assuming that each light chain encompasses 50% of the lever length, then an $L$ value of 0.0, 0.5, and 1.5 were assigned to neckless, -R site, and CABL-giraffe, respectively. The linear regression for the data does not pass through zero but intersects the $x$ axis at $-0.34L$, suggesting that the lever may extend $-3 \text{ nm}$ (i.e. 0.34 ± 0.5 nm neck length) into the motor domain.
other neckless myosin species created genetically in actuated significant force and motion. Motion was also observed for tend into the motor domain, since our neckless mutant gener-
leva arm length is linear, strongly supporting the hypothesis control. The relationship between unitary displacements and mutant generated displacements that were 1.4 times that of its lever arm length. Unitary displacements of two short necked the laser trap, the magnitude of displacements, and unitary forces. If the neck acts as a simple model, that we previously proposed to explain the force- and motion-generating capacity of light chain-deficient skeletal myosins (10, 30), predicts that force should be linearly related to lever arm length. The solid lines are the predicted relationships for the depend-
S.E. For these data, n indicates the number of independent data sets analyzed by MV analysis (see "Experimental Procedures"). ND, not determined.

| TABLE I  
| Summary of mechanical data |
| Construct | Motility, v_{max} | Laser trap | P | n |
|           | μm/s            | d | t_m | n | F | n |
| Short neck constructs |          |    |     |   |   |   |
| Control |          |    |     |   |   |   |
| WT HMM | 1.1 ± 0.19 (4) | 10.5 ± 0.8 | 196 ± 50 | 5 | 1.6 ± 0.3 | 5 |
| Short necks |          |    |     |   |   |   |
| Neckless | 0.26 ± 0.04 (3) | 2.0 ± 0.6^a | ND | 5 | 1.1 ± 0.7 | 2 |
| - R site | 0.49 ± 0.18 (2) | 6.2 ± 1.2^a | 45 ± 6 | 6 | ND |   |
| Long neck constructs |          |    |     |   |   |   |
| Control |          |    |     |   |   |   |
| CABL-HMM | 0.45 ± 0.08 (4) | 9.1 ± 0.8 | 64 ± 5 | 10 | 0.9 ± 0.4 | 3 |
| Long necks |          |    |     |   |   |   |
| CABL-giraffe | 0.42 ± 0.08 (4) | 12.4 ± 0.6^b | 85 ± 6 | 14 | 2.1 ± 0.4 | 4 |

^a Significantly different from WT HMM.
^b Significantly different from CABL-HMM.

mately twice that generated by CABL-HMM (Table I). Given the higher force-generating capacity of the CABL-giraffe, it is unlikely that the addition of an ELC site introduced a significant compliance within the neck, which potentially could have explained why the rate of motility of CABL-giraffe was not faster than its control CABL-HMM.

**DISCUSSION**

The goal of this study was to provide a stringent test for whether the myosin neck region acts as a mechanical lever that transmits force and displacements originating within the motor domain. We therefore characterized the molecular mechanics of smooth muscle HMM mutants with shorter and longer neck regions by measuring in vitro motility velocities, unitary displacements, and unitary forces. If the neck acts as a simple lever arm, one prediction is that under unloaded conditions in the laser trap, the magnitude of d should be directly related to lever arm length. Unitary displacements of two short necked constructs were 20% (neckless) and 60% (-R site) of the values obtained with WT HMM. Strikingly, the long necked giraffe mutant generated displacements that were 1.4 times that of its control. The relationship between unitary displacements and lever arm length is linear, strongly supporting the hypothesis that the neck acts as a lever arm (Fig. 6).

Another conclusion of this study is that the lever must extend into the motor domain, since our neckless mutant generated significant force and motion. Motion was also observed for other neckless myosin species created genetically in *Dictyostelium* myosin (27) or proteolytically from skeletal muscle myosin (28). The portion of the long a-helix that remains in the neckless construct (residues 778–790), which abuts the more compact domain of the converter region (residues 721–777), could provide an additional piece of lever arm for generating force and motion in this construct. The crystal structures of skeletal subfragment 1 and smooth muscle MDE show that the rotation of the converter and the lever arm occurs because of two perpendicular rotations around two conserved glycines that are located at either end of the SH1 helix (4). In agreement with the structural data, the correlation between neck length and d (Fig. 6) suggests that the fulcrum for movement is ~3 nm within the motor domain. Therefore, the bulk of the motor domain remains in a relatively fixed orientation with respect to actin, and the point at which myosin undergoes a major rotation is closer to the converter region than to the actomyosin interface.

The lever hypothesis also makes specific predictions about the relationship between lever arm length (L) and unitary force (see Fig. 7), but these are model-dependent. In contrast, all three models described below predict a linear relationship between lever arm length and unitary displacement (Fig. 6).

![Image](345x382 to 517x519)

**Fig. 7. Model relationships for the effect of lever arm length on force.** Unitary forces (∂) for neck length mutants normalized to their respective controls (i.e. WT HMM for neckless and CABL-HMM for CABL-giraffe) are plotted as mean ± S.D. against relative lever arm length. The solid lines are the predicted relationships for the depend-
educity of unitary force on lever arm length (see “Discussion” for details). #1, the prediction for a simple lever (i.e. $F \propto L^{-1}$); #2, the prediction (i.e. $F \propto L^{-2}$) based on the model of Howard and Spudich (Appendix in 13); #3, the prediction (i.e. $F \propto L$) based on the model of Van Buren et al. (30). The mutant data are offset on the x axis by 0.54 lever arm lengths based on the prediction in Fig. 6 as to the origin of the lever arm. The mutant data are best fit by model 3, where the neck region acts like a rigid lever that is coupled to a torque motor within the motor domain and to an elastic element presumably in the subfragment 2 segment (30).
presumably in the subfragment 2 region (30). A comparison of the force data from the laser trap with the predictions for the three models (see Fig. 7) suggests that the third model may be the most appropriate. Both the displacement and the force data support the conclusion that the neck region acts as a rigid lever and that much of the myosin elasticity is external to the neck. It should be noted that force measurements in this assay are underestimates, given the stray compliances within the experimental system (see “Experimental Procedures”), and thus conclusions based on these data should be viewed in light of this concern.

An alternative view of the neck has been championed by Yanagida and co-workers (31). Although they also obtained a reduced velocity with a similar neckless construct of Dictyostelium myosin, they showed that the unitary step size was unaltered in these smooth muscle myosin constructs, where myosin activity is tightly regulated by light chain phosphorylation. Thus, the neck region acts as a lever arm but may also serve to transmit mechanical strain to the catalytic site within the motor domain.

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REFERENCES

1. Rayment, I., Rypniewski, W. R., Schmidt-Baue, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) Science 261, 59–58
2. Rayment, I., Holden, H. M., Whitaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1995) Science 261, 65–65
3. Fisher, A. J., Smith, C. A., Thoden, J., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) Biophys. J. 68, 195–265
4. Dominguez, R., Freyzon, Y., Trybus, K. M., and Cohen, C. (1998) Cell 94, 579–571
5. Finer, J. T., Simmons, R. M., and Spudich, J. A. (1994) Nature 368, 113–119
6. Molloy, J. E., Burns, J. E., Kendrick-Jones, J., Tregear, R. T., and White, D. C. S. (1995) Nature 378, 209–212
7. Guilford, W. H., Dupuis, D. E., Kennedy, G., Wu, J., Patlak, J. B., and Warshaw, D. M. (1997) Biophys. J. 72, 1006–1021
8. Kitamura, K., Tokunaga, M., Iwane, A. H., and Yanagida, T. (1999) Nature 397, 129–134
9. Block, S. (1996) Cell 87, 151–157
10. Lowey, S., Waller, G. S., and Trybus, K. M. (1993) Nature 365, 454–456
11. Trybus, K. M. (1994) J. Biol. Chem. 269, 20819–20822
12. Uyeda, T. Q. P., and Spudich, J. A. (1993) Science 262, 1867–1870
13. Uyeda, T. Q., Abramson, P. D., and Spudich, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4459–4464
14. Anson, M., Geeves, M. A., Kurzawa, S. E., and Manstein, D. J. (1996) EMBO J. 15, 6069–6074
15. Huxley, H. E. (1990) J. Biol. Chem. 265, 8347–8350
16. Rovner, A. S., Freyzon, Y., and Trybus, K. M. (1995) J. Biol. Chem. 270, 30260–30263
17. White, H. D. (1982) Methods Enzymol. 85, 698–708
18. Trybus, K. M., and Lowey, S. (1984) J. Biol. Chem. 259, 8564–8571
19. Craig, R., Szent-Gyorgyi, A. G., Beese, L., Flicker, P., Vibert, P., and Cohen, C. (1980) J. Mol. Biol. 140, 35–55
20. Trybus, K. M., and Chatman, T. A. (1993) J. Biol. Chem. 268, 4412–4419
21. Trybus, K. M., and Henry, L. (1989) J. Cell Biol. 109, 2879–2886
22. Work, S. S., and Warshaw, D. M. (1992) Anal. Biochem. 202, 275–285
23. Dupuis, D. E., Guilford, W. H., Wu, J., and Warshaw, D. M. (1996) J. Muscle Res. Cell Motil. 18, 17–30
24. Lauzon, A.-M., Tyska, M. J., Rovner, A. S., Freyzon, Y., Warshaw, D. M., and Trybus, K. M. (1998) J. Muscle Res. Cell Motil. 19, 825–837
25. Patlak, J. B. (1993) Biophys. J. 65, 29–42
26. Tyska, M. J., Dupuis, D. E., Guilford, W. H., Patlak, J. B., Waller, G. S., Trybus, K. M., Warshaw, D. M., and Lowey, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4402–4407
27. Itakura, S., Yamakawa, H., Toyoshima, Y. Y., Ishijima, A., Kojima, T., Harada, Y., Yanagida, T., Wakahayashi, T., and Sutoh, K. (1995) Biochem. Biophys. Res. Commun. 196, 1504–1510
28. Waller, G. S., Ouyang, G., Swafford, J., Vibert, P., and Lowey, S. (1995) J. Biol. Chem. 270, 15348–15352
29. Dohle, I., Linari, M., Piazzesi, G., Reconditi, M., Koubassova, N., Ferenczi, M. A., Lombardi, V., and Irving, M. (1998) Nature 396, 383–387
30. VanBuren, P., Waller, G. S., Harris, D. E., Trybus, K. M., Warshaw, D. M., and Lowery, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12403–12407
31. Tanaka, H., Kitamura, K., Iwane, A. H., and Yanagida, T. (2000) Biophys. J. 78, 3A (abstr.)
32. Chaudoir, B. M., Kowalczyk, P. A., and Chisholm, R. L. (1999) J. Cell Sci. 112, 1611–1620
33. Diffee, G. M., Patel, J. R., Reinach, F. C., Greaser, M. L., and Moss R. L. (1996) Biophys. J. 71, 341–350