Self-organization of actin networks by a monomeric myosin

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The organization of actomyosin networks lies at the center of many types of cellular motility, including cell polarization and collective cell migration during development and morphogenesis. Myosin-IXa is critically involved in these processes. Using total internal reflection fluorescence microscopy, we resolved actin bundles assembled by myosin-IXa. Electron microscopic data revealed that the bundles consisted of highly ordered lattices with parallel actin polarity. The myosin-IXa motor domains aligned across the network, forming cross-links at a repeat distance of precisely 36 nm, matching the helical repeat of actin. Single-particle image processing resolved three distinct conformations of myosin-IXa in the absence of nucleotide. Using cross-correlation of a modeled actomyosin crystal structure, we identified sites of additional mass, which can only be accounted for by the large insert in loop 2 exclusively found in the motor domain of class IX myosins. We show that the large insert in loop 2 binds calmodulin and creates two coordinated actin-binding sites that constrain the acto-myosin interactions generating the actin lattices. The actin lattices introduce orientated tracks at specific sites in the cell, which might install platforms allowing Rho-GTPase-activating protein (RhoGAP) activity to be focused at a definite locus. In addition, the lattice tracks might introduce a myosin-related, force-sensing mechanism into the cytoskeleton in cell polarization and collective cell migration.

Significance

Actomyosin networks are central to a broad range of cellular motile processes, including cell polarization and collective cell migration during morphogenesis and development. Myosin-IXa is critically involved in these processes. Using fluorescence spectroscopy, total internal reflection fluorescence, and electron microscopy, we demonstrate that myosin-IXa assembles actin filaments into highly ordered lattices. The actin filaments of parallel polarity are connected by myosin-IXa in distinct conformations and at a repeat distance of 36 nm across the network. The myosin-IXa-induced actin lattices introduce orientated actin tracks and a network of regularly spaced platforms for localized Rho-GTPase-activating protein activity in cell polarization and collective cell migration.


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absence and in the presence of ATP. By inducing the formation of highly ordered actin lattices, the myosin-Ixα motor domain (IXα-MD) might install platforms for localized RhoGAP activity and introduce specific tracks for other myosin motors into the actin cytoskeleton in cell polarization and collective cell migration.

**Results**

**The Motor Domain of Myosin-Ixα Binds Calmodulin.** The detailed structure of class IX myosins with their unique insert in the catalytic domain is unknown. To address this problem, we expressed the human IXα-MD, including the unique ~207-aa insert into loop 2 (MD-CBS) (Fig. 1A, SI Text, and Fig. S1 A–D). Sequence analysis indicated a 1-8-14 calmodulin-binding site (CBS) at the N terminus of the loop 2 insert (MD-CBS) (Fig. S1E) (15). Consistent with this prediction, we found that the IXα-MD copurified with calmodulin at a 1:1 molar ratio in the absence and presence of calcium (Fig. 1B and Fig. S1C). Solution kinetics studies revealed that, in the presence of calcium, the ATPase of the motor domain was activated by a factor of 5 by actin, whereas the motor domain generated actin movement of ~27 nm·s⁻¹ in gliding-filament assays (Fig. S1F), which confirmed that the motor domain was enzymatically and mechanically active. The data also indicated that the activity of myosin-Ixα was regulated by calcium–calmodulin binding to the motor domain (Fig. 1C). We then investigated the structural effect of calmodulin binding to the motor domain in further detail using the intrinsic tryptophan fluorescence of the target peptide MD-CBS in loop 2 (SI Text) (22). We chose calcium concentrations close to the physiological range (~50 nM to ~10 μM), which ensured homogeneous populations of the molecules (23–28). The blue shift of the emission spectrum and increase in tryptophan fluorescence intensity at λ emission (λem) 323 nm upon calcium–calmodulin binding to the peptide reached saturation at a 1:1 binding stoichiometry (dashed line) and yielded a binding constant K_d of 55 nM (Fig. 1D and E). The binding studies indicated that calmodulin was binding the target peptide fully reversibly in different conformations in the presence and absence of calcium, consistent with calmodulin remaining bound to IXα-MD (Fig. 1F). Given that calmodulin was binding to the N-terminal part of loop 2, a region previously shown to contribute to the actin-binding site on the myosin motor domain (11, 12), the question arose whether myosin-Ixα binding to actin was substantially different from that of other classes of myosin, which would have strong implications on its molecular mechanisms (Fig. 1G).

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**Fig. 1.** The IXα-MD binds a calmodulin in the presence and absence of calcium. (A) Domain structure of myosin-Ixα, including the motor domain (gray), N-terminal extension (white), and CBS motif in loop 2 (MD-CBS) and the neck domain (yellow) with six calmodulin (CaM)-binding motifs, followed by the tail domain (white). The IXα-MD construct comprises the motor domain without the N-terminal extension. (B) SDS/PAGE of expressed IXα-MD confirms clean protein that copurified with CaM. Note the electrophoretic mobility shift of CaM in the presence of calcium (blue arrows). (C) Actin-activated ATPase of 100–200 nM IXα-MD at 2 mM ATP in the presence and absence of calcium. Fitting to Michaelis–Menten kinetics in EGTA: V_o = 0.12 ± 0.01 s⁻¹, V_max = 0.49 ± 0.03 s⁻¹, and K_M = 2.0 ± 1.3 μM. (D) Tryptophan fluorescence (λ_em 290 nm) for 2 μM target peptide IXα-MD–CBS and increasing concentrations of CaM (0–4 μM) measured at pCa 4. (E) IXα-MD–CBS bound to CaM with a 1:1 stoichiometry and a K_d of 55 nM at pCa 4. The linear increase in fluorescence in the higher concentration range was due to increasing CaM dominating the fluorescence signal (dotted line). (F) The change in tryptophan fluorescence (λ_em 290 nm; λ_ex 323 nm) in response to a change in calcium concentration, measured with 2 μM IXα-MD–CBS and 2 μM CaM, showed that CaM binding to IXα-MD–CBS was fully reversible and calcium-dependent. Black line, pCa ≥8; red line, pCa 4. (G) The cartoon illustrates calcium–CaM binding to IXα-MD–CBS in loop 2 of the IXα-MD.
**IXa-MD Cosediments with Actin in the Absence and Presence of ATP.**

We investigated the ability of the IXa-MD to bind to actin filaments in different nucleotide conditions and performed cosedimentation experiments in which IXa-MD was mixed with increasing actin concentrations in the presence and absence of nucleotide. A complete binding of IXa-MD was observed in the nucleotide-free state. In the presence of ADP and ATP, however, the affinity for actin dropped only by a factor of ~2, independent of calcium, which—in particular in the ATP-bound state—indicated a remarkably high actin affinity for a myosin motor (Fig. 2 A–C), as reported previously for other myosin-IX isoforms (17). The fact that both ADP and ATP only reduced the fraction of IXa-MD bound to actin by ~50% implied that there was an equilibrium between a fraction of myosin-IXa that still bound to actin and another one that did not. When the IXa-MD population from the supernatant obtained in the presence of ATP was mixed with filamentous (F)-actin and resedimented, the two subpopulations of IXa-MD were observed again, which indicated that the two subpopulations interconverted on the time scale of the cosedimentation experiments (Fig. S1G).

The cosedimentation with actin in the presence of ATP, combined with the reported processivity of single motor domains of other myosin-IX isoforms (9, 10, 13–16), leads to the following question: Does myosin-IXa contain a second actin-binding site in the motor domain which is able to cross-link actin filaments?

**Formation of Actin Bundles Is Induced by IXa-MD.**

We tested the cross-linking hypothesis by quantitatively analyzing total internal-reflection fluorescence (TIRF) microscopy data, using Alexa-Fluor488–labeled actin filaments and mutant or wild-type myosin-IXa (Fig. 3). We first investigated the effect of a mutant construct with the myosin-IXa–specific loop 2 exchanged for a human skeletal muscle loop 2. Even at a 1:1 molar ratio of mutant IXa-MD to actin and in the absence of ATP, no bundling was observed (Fig. 3A). In contrast, at the same molar ratio, wild-type IXa-MD induced the formation of actin bundles in the absence and presence of ATP (Fig. 3B and Fig. S2). The bundles were characterized by analyzing the fluorescence intensity along single-actin bundles (Fig. 3C) and by analyzing the total pixel intensity across 100 fields of view (600 × 600 μm²) in total (Fig. 3D). The stepwise increase in fluorescence intensity along individual actin bundles and the quantized distribution of total fluorescence intensity across 100 fields of view were consistent with up to 7 or more actin filaments forming bundles in the absence and presence of ATP. The formation of bundles was fully reversible and dependent on actin, myosin, and ATP concentrations. At 1 μM actin and a 1:1 molar ratio of actin to IXa-MD bundling was still observed at 0.25 mM ATP (Fig. S2 A and B). To understand the molecular mechanisms that enabled the IXa-MD to induce the dynamic formation of actin bundles, we investigated the structures of these myosin-IXa cross-links using EM.

**IXa-MD Cross-Links Actin at a Repeat Distance of 36 nm to Form Lattices.**

Negative stain EM of the actin bundles showed that the bundles consisted of regular networks formed by myosin-IXa cross-linking up to five actin filaments (Fig. 4A). The bundle size was limited in these experiments by the necessity to dilute the sample for EM. Remarkably, the myosin cross-links aligned across the network to generate regular lattice structures (Fig. 4B, yellow arrows). The class average of 981 images in Fig. 4C revealed that IXa-MD formed cross-links between actin filaments with a periodicity of 36 nm. IXa-MD binding to noncross-linked single-actin filaments was never observed, nor did we find extra molecules in between the 36-nm pattern, strongly suggesting that binding was highly cooperative between the two actin-binding sites on the IXa-MD.

**IXa-MD Adopts Three Distinct Conformations and Binds to Actin Filaments with Parallel Polarity.**

The shape of the averaged actin cross-link comprised a central mass connected to a total of 4 actin monomers, including two adjacent monomers on either actin filament (Fig. S4 and SI Text). Classification of the 981 images of IXa-MD according to the myosin connections to the four actin monomers unveiled three distinct conformations, two of which formed a cross-link between the actin filaments (Fig. S5B and Fig. S3). At 44%, the diagonally shaped cross-link (conformation I) was the most frequently observed, followed by the bent cross-link (conformation II) at 29%. In the remaining inchworm conformation (27%), the myosin molecules did not cross-link but formed a bridge between two adjacent actin monomers on the same actin filament (conformation III). To resolve the polarity of
the actin filaments cross-linked by myosin-IXa, we generated model structures of two actin filaments with either parallel or antiparallel polarity and with the actin pitch either in phase or out of phase (Fig. S5C and SI Text). Cross-correlation of the real-EM images with the low pass-filtered actin models divulged that 97% of the myosin cross-links were formed between two parallel actin filaments aligned in phase (Fig. 5 C and D). Analysis of the variance between the images localized the hotspots of variability at the connection between the central myosin mass—bound to one actin monomer—and a second actin monomer, either on the same or on the other actin filament, which was consistent with the image classification into three myosin-IXa conformations [i.e., into the diagonal (I), the bent (II), and the inchworm conformation (III)] (Fig. 5D, pink spots, and Fig. 5E, white circles). To interpret the EM data of the actin cross-links, we fitted a modeled crystal structure of an actomyosin-IE complex to the data (29) (Fig. S4A–D). Fig. 5E shows that the actomyosin-IE complex, overlaid onto the averaged real EM in an optimized projection [cross-correlation coefficient (ccc): 0.63; Fig. S4], could account for both the actin monomers on the bottom actin filament (green) and for the central mass of the myosin-IXa cross-links (blue). The three hot spots of variability in the EM average, linking the central myosin mass to each of the three actin-binding sites, were not covered by the model structure and therefore probably belong to the remaining part of the loop 2 insert in myosin-IXa. To include the calmodulin on loop 2, we fitted a calmodulin–peptide complex by extending the α-helix preceding the N terminus of loop 2 in the myosin-IE structure (Fig. 5E, asterisk). The inserted calmodulin was consistent with the additional mass next to the myosin catalytic domain in the EM data not directly connected to the actin filaments. The cross-linking connections to the second actin-binding sites in conformation I and II and the inchworm connection in conformation III (white circular outlines), however, were not accounted for by the model structure and are therefore probably due to the additional ∼200 aa present in loop 2 of the myosin-IXa head domain.

**Discussion**

A central feature of many cellular motile processes lies in the local organization of actomyosin networks. The molecular mechanisms of chemomechanical energy transduction of several myosin motors with very diverse mechanical and kinetic properties have been investigated at the single-molecule level (30, 31). However, the mechanisms organizing the local actomyosin networks, including those involved in cell polarization and during collective cell migration, remain unclear. Here, we discovered that the catalytic domain of myosin-IXa self-organizes regular actin networks in the shape of extended lattices. We discovered that this finding was due to the presence of a large insert of ∼207 aa in loop 2 of the catalytic domain of the myosin-IXa motor.

We found that a single calmodulin bound to the CBS motif at the N terminus of loop 2 in the IXa-MD, which is consistent with recent reports for other myosin-IX isoforms (10, 15). Calmodulin binding was observed in the absence and presence of calcium, albeit in two different conformations, which suggests that calcium–calmodulin on the motor domain might play a regulatory role for the myosin-IXa motor molecule. The switch from a high-affinity ($K_d$, 55 nM) 1:1 calmodulin to peptide stoichiometry at high calcium to an undetermined stoichiometry at low calcium suggests that at low calcium, additional CBSs located in other regions of the loop 2 insert or even outside loop 2 might become involved to restore the correct configuration (23). A regulatory effect of calcium–calmodulin was consistent with the myosin-IXa ATPase, which was actin activated in the presence of calcium by a factor of 5. The maximum rate of ∼0.5 s$^{-1}$ was similar to previous reports on motor domain constructs of another myosin-IX isoform in *Caenorhabditis elegans* (15). The fact that actin activation by a factor of 5 was observed for the myosin-IXa...
construct in the presence and for the *C. elegans* isoform in the absence of calcium (15) might be explained by differences in the sequence of the CBS motif and in the remainder of the loop 2 insert in these myosin-IX isoforms, which are considerably different in size (Fig. 1 and Fig. S1).

The large ∼100- to 200-aa insert into loop 2, including the CBS motif, is a distinguishing feature of class IX myosins (15). We show that the modification of loop 2, which in other classes of myosin is involved in actin binding (11), not only features a CBS in myosin-IXa but also retains an unusually high actin affinity, specifically in the ATP-bound state of this myosin motor. A fraction of 0.6 of the IXa-MD cosedimented with F-actin at millimolar ATP concentrations, consistent with fractions of 0.24–0.9 reported previously for other myosin-IX isoforms, depending on the specific constructs and conditions used (10, 16, 17). This result indicated that the unusually high affinity for actin in the presence of ATP is a feature of the loop 2 insert of myosin-IXa and does not require the presence of other structural parts of the molecule, such as an N-terminal extension. Previous studies on other myosin-IX isoforms suggested that the high affinity for actin enables the loop 2 insert to act as an actin tether that prevents monomeric myosin-IX from dissociating from actin in the ATP-bound state during processive movement (10, 13–16, 32). We discovered that the insert’s high affinity for actin enables myosin-IXa to cross-link and bundle actin filaments.

The fluorescence studies showed that the loop 2 insert in myosin-IXa was in fact the molecular basis for myosin’s actin-cross-linking properties and the formation of large actomyosin bundles consisting of seven or more actin filaments. We found that this feature of a myosin catalytic domain comprises the fully reversible assembly and disassembly of actin bundles, dependent on the concentration of available actin filaments and on ATP. At higher ATP concentrations, smaller bundles were observed. Technical reasons required the actin to be kept at 1 μM, two orders of magnitude below the physiological intracellular concentration range [on average, ∼100 μM (33)], which limited the availability of actin filaments for dynamic assembly/disassembly by myosin-IXa into bundles, in particular, in the presence of ATP. Nevertheless, actin bundles still formed in the presence of up to 0.25 mM ATP (Fig. S2), confirming the high propensity of this motor to induce actin bundles.

It remains to be clarified how the assembly and disassembly of the actin lattices by myosin-IXa are regulated. The ability to discern bundle thickness using quantitation of the fluorescence signal enabled us to monitor the disassembly of larger bundles into smaller ones and the detachment of single-actin filaments from bundles (Fig. S2C). These observations were consistent with a mechanism in which preformed actin filaments are reversibly zipped up into bundles, regulated by the local availability and recruitment of myosin-IXa. The pull-down experiments at higher actin concentrations, closer to the physiological range, showed 40–80% of IXa-MD bound to actin at millimolar ATP or ADP concentrations, consistent with a regulatory role of nucleotide for the actin-bundle formation in the cell. As described for myosin-V (34, 35) the nucleotide states of myosin-IXa themselves might be affected by load regulating the structure of the actin bundles in a force-dependent fashion. Another regulatory candidate might be calcium–calmodulin bound to the insert in loop 2. In the pull-down experiments, the propensity of IXa-MD to induce actin bundles was unaffected by calcium, consistent with the EM data that confirmed the formation of IXa-MD cross-links at the repeat distance of 36 nm at pCa 4.0 (Fig. S2D).

These cross-links were again consistent with the three IXa-MD conformations—diagonal, bent, and inchworm—as observed at
low calcium conditions (pCa 8.0). However, the dynamic equilibrium between the different conformations in different regulatory calcium and nucleotide conditions remains to be established. Finally, for full-length myosin-IXa, a further regulatory candidate might be the Rho-GAP domain in the tail, possibly affecting both actin filament formation and the structure of the actin bundles.

The novelty for myosin-IXa is the presence of two separate actin-binding sites in the catalytic domain that enable this myosin motor to cross-link and orientate actin filaments and to induce the formation of highly regular actin lattice structures. The cross-linking property of the catalytic domain is based on a remarkable structural flexibility that enables the loop 2 insert to attach to three different actin-binding sites, forming either two types of cross-links bridging across two different actin monomers or forming an inchworm conformation on the same actin filament. The detailed structure of the loop 2 insert is currently unknown. However, studies on the myosin-IXb loop 2 have reported it to contain a substantial amount of secondary structure but to remain flexible with elastic properties and a high affinity for actin (10). In striking contrast to the cross-links observed with single-headed myosin I isoforms, which contain a second actin-binding site in the tail domain of the motor molecule (36, 37), we found that myosin-IXa induced actin bundles by forming highly regular binding patterns, cross-linking the actin filaments at a repeat distance of 36 nm matching the actin helical repeat, and leading to an alignment of myosin cross-links throughout the actin network. Intriguingly, we did not observe single-actin filaments decorated with IXa-MDs. This result suggests that the myosin-IXa binding to actin is highly cooperative and involves two actin-binding sites on the motor domain and a dynamic equilibrium between the inchworm (III) and the two cross-linking (I and II) conformations. Binding positions corresponding to the 36-nm actin helical repeat where myosin-IXa can adopt a maximum number of binding conformations seem to be strongly favored. ATP-dependent inchworm-type processive movement of myosin-IXa along single-actin filaments would not

Fig. 5. Interpretation of IXa-MD-induced actin cross-links using a modeled crystal structure. (A) Class average of 981 actomyosin-IXa cross-links. The polarity of the actin filaments was not taken into account. (B) The classification revealed three different conformations; the frequency of observation is given in percentage. (C) Cross-correlation with low pass-filtered parallel and antiparallel in and out of phase actin models showed that 97% of the cross-links were formed between parallel actin filaments, aligned in phase (polarity 1, 2.2% polarity 2, 0.4% polarity 3 and 4). The fins in the cartoons of two actin filaments indicate the polarity and phase. (D) Realigned class average (inverted) of the 221 cross-links between parallel actin filaments aligned in phase. The variance analysis between the realigned cross-links resulted in three hotspots (pink), indicating three secondary myosin-binding sites on actin, consistent with the three myosin conformations identified in B. (E) The IXa-MD cross-links were interpreted using an actomyosin-IIE structure, including a calmodulin modeled onto loop 2. The optimized projection of the model was overlaid onto the real-EM class average (actin monomers, green; myosin motor domain, blue; calmodulin bound to loop 2, yellow). The circles mark the hotspots of variance seen in D, and the roman numerals denote the different conformations shown in B.
be required to generate actin lattices in this model. However, we propose that in the inchworm conformation, myosin-IXa might be able to proceed processively along a single-actin filament, as had been reported for other myosin-IX isoforms (9, 10, 13–16), to seek out the next preferred binding position, where a second actin filament comes into reach, so that both the inchworm and the cross-linking conformations can be adopted; otherwise, the actin binding of this particular motor might not be sustained. So far, we did not observe processive movement of single IXa-MDs along single-actin filaments, which we investigated using motility assays and single-molecule mechanical experiments (SI Text). This result may be due to either very short processive run lengths or some unfavorable interactions of IXa-MD with its larger insert compared with other myosin-IX isoforms, or the result might reflect differences in the structure of the loop 2 insert and possibly the distribution and function of the myosin-IXa isoform.

The three different myosin-IXa conformations obtained in the absence of ATP do not seem to represent different nucleotide states but rather three different states in nucleotide-free rigor at the end of the chemomechanical cycle. The difference in the frequency of observation indicated different binding affinities in the different conformations, possibly due to intramolecular strain, with bent cross-links experiencing a larger strain than diagonal ones. Future experiments could resolve the effect of nucleotide on the different conformations.

We propose the following model for the formation of lattice-like actomyosin networks induced by myosin-IXa (Fig. 6): (i) the IXa-MD binds to an actin filament with one of the two actin-binding sites in loop 2; (ii) if a second actin filament of the same polarity comes within reach, the IXa-MD adopts a diagonal or a bent cross-link (conformations I and II); the cross-link aligns the two actin filaments in parallel polarity and in phase; and (iii) additional IXa-MDs are now recruited at the preferred binding positions along actin, determined by the 36-nm periodicity and by the spacing in between the actin filaments; at the preferred binding positions, myosin-XIa can adopt a maximum number of binding conformations (inchworm as well as diagonal and bent cross-links), which enforces the formation of an actin lattice structure. ATP-dependent inchworm-type processive movement of myosin-IXa along single-actin filaments would not be required to generate actin lattices in this model. However, processive movement might promote myosin-IXa seeking out the next preferred binding position with the option to cross-link actin filaments. Future experiments could investigate the mechanical properties of myosin-IXa in these networks in further detail. By inducing the formation of highly ordered actin lattices, myosin-IXa might provide specific tracks for other myosin motors and install platforms, allowing RhoGAP activity at the myosin-IX tail to be located at definite loci. In addition, the regularly spaced, cross-linking myosin motors in the lattice of oriented tracks might introduce a novel, cooperate force-sensing mechanism into the cytoskeleton in cell polarization and collective cell migration.

Materials and Methods

Molecular Biology. Full-length human myosin-IXa (7,647 bp) cDNA (National Center of Biotechnology accession no. AA40870.1) was codon-optimized, chemically synthesized, and cloned into plasmid University of California 57 (pUC57) (GenScript). This construct was used as a template to subclone the IXa-MD (base pairs 445–3,057) construct using standard PCR methods and oligonucleotides via BamHI/XbaI sites into the pFBN vector, with MD9a-forward (fwd) (GGATCCATGTGATTTATTTATGCTGTTGAACACCATATG) and 1019-reverse (rev) (GGCCGCTTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCATC) as primers. The reverse primer contained a sequence encoding the FLAG-tag (DYKDDDK) upstream of the stop codon and XbaI site. Introduction of an AviTag between the end of the myosin-IXa sequence and the FLAG-tag was achieved via a ligation at the NotI site using chemically synthesized oligonucleotides with free phosphate groups at the 3′-end Avi-tag-fwd (GGCCGCCAGGTGGGCTCTCCACCGGCATGGCTCAAGAACATCTTCAGGCTCAGAAAGATTCGAAGTGGCGACGAAAG) and Avi-tag-rev (GGCCGCATCTGGCACTTGTTCTGATCTGGCAGTAGCTGGTACAAGCGGCACCTTG). Recombinant baculovirus (Bac) DNA was generated by the Bac-to-Bac (Invitrogen) method according to the manufacturer’s instructions and transferred into SF21 cells. Viruses were amplified to a P3 stock, and virus titer was determined before infection of cells for protein expression.

Tryptophan Fluorescence. The tryptophan fluorescence studies were performed with the target peptide sequence from human myosin-IXa [National Center of Biotechnology accession no. NM_006901.2; the peptide sequence shown in Fig. 5 (green) was synthesized by GenScript and human calmodulin. The titrations of the predicted target peptide (38, 39) with calmodulin were performed and analyzed at 20 °C in the following buffer: 25 mM Tris (pH 8.0), 100 mM KCl, 1 mM DTT, supplemented with either 1 mM CaCl2 or 0.2 mM EDTA, using a Varian Cary Eclipse fluorescence spectrophotometer (λ excitation 290 nm; λ emission 323 nm), as described (22, 40). The dissociation constants Kd for the tryptophan-containing peptide were determined by direct titration, as described previously (22). The equations used to fit the data are described in SI Text.

Steady-State Mg-ATPase. The experiments were performed in a buffer containing 10 mM 3-(N-morpholino)propanesulfonic acid (Mops) (pH 7.2–7.4), 50 mM KCl, 0.1 mM EGTA, 1 mM MgCl2, 1 mM DTT, and 2 μM calmodulin, using a NADH-coupled enzyme assay at 22 °C and a Varian Cary 50 spectrophotometer as described (17, 41). The buffer was supplemented with 1 mM phosphoenolpyruvate, 0.2 mM NADH, 18 U/ml lactate dehydrogenase, 12 U/ml pyruvate

Fig. 6. Model. Actin filaments with parallel polarity are cross-linked by the motor domains of single myosin-IXa molecules adopting three interchangeable conformations (I, II, and III) (Inset) that can be obtained at binding sites with free-energy minima. IXa-MD does not remain bound to single-actin filaments, and the precise 36-nm spacing is obtained due to the constraints set by the length and flexibility of loop 2 and the distances between actin filaments specified by their geometries.
kinase, 2 mM ATP, and a range of phalloidin-stabilized F-actin concentrations. The reaction was started by adding 0.1–0.2 μM IXa-MD, and the decrease in absorbance at 340 nm (ΔA340), the myosin concentration in the reaction and the NADH extinction coefficient (ε = 6220 M\(^{-1}\) cm\(^{-1}\)) as described (17). The ATPase rate of F-actin alone was subtracted from the actomyosin ATPase rate. The measurements were repeated with 3 different myosin preparations for each actin concentration and the data were fitted to the Michaelis-Menton equation (SI Text).

Cosedimentation of the IXa-MD with F-Actin. Cosedimentation assays were performed with IXa-MD and increasing concentrations of actin in the presence and absence of nucleotides as described (17). In brief, actin was polymerized in 25 mM Mops (pH 7.2), 50 mM KCl, 2 mM MgCl\(_2\), 1 mM DTT, and 1 mM EGTA, and filaments were stabilized by adding phalloidin at a 1:1 molar ratio; 0.2–0.5 μM IXa-MD was incubated with 0.15–0.5 μM F-actin in the absence or presence of nucleotide in a buffer containing 10 mM Mops (pH 7.2), 100 mM KCl, 4 mM MgCl\(_2\), 1 mM DTT, and 0.1 mM EGTA. Apyrase was used to deplete the solution fully from nucleotide in experiments in the rigor condition. Following 20 min of centrifugation at 435000 × g and 4 °C, pellets were resuspended in an equal volume of phosphate buffer, and equal amounts of supernatants and pellets were analyzed by SDS/PAGE. For each experiment, myosin in the supernatants and in the pellets was quantified and normalized by densitometry. The dissociation constants Kd of myosin binding to actin were determined by fitting to the solution of the standard quadratic equation (17), as described in SI Text.

TIRF Microscopy. TIRF imaging was performed on a Nikon (Eclipse Ti) microscope. F-actin was stabilized with Alexa-Fluor488-phalloidin (Molecular Probes) at a 1:1 molar ratio and mixed with IXa-MD also at a 1:1 molar ratio. To image and analyze the IXa-MD–actin bundles, they were introduced into a flow cell coated with 10 μg/ml N-ethylmaleimide–modified myosin (42) in an assay buffer containing 10 mM Mops (pH 7.2), 250 mM NaCl, 0.1 mM EGTA, mM 10 DTT, 3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, and ATP as indicated. For the automated image analysis, 100 adjacent fields of view covering a total area of 600 × 600 μm\(^2\) were recorded by systematically translating the microscope stage. To ensure homogeneous conditions of fluorescence excitation and imaging, a central region of interest (ROI) (60×60 μm) of the image was identified within the image was identified by dividing by a reference image of the laser-intensity distribution. Based on the signal intensity of all pixels within the ROI, the calculated total length of actin bundle structures consisting of one to seven actin filaments.

Negative-Stain EM. Nucleotide-free IXa-MD and F-actin were diluted to a final concentration of 1 μM and 0.5 μM, respectively, using a buffer containing 20 mM Mops (pH 7.2), 100 mM NaCl, 0.2 mM EGTA, and 1 mM DTT. The mixed protein sample was applied to hydrophilized (glow-discharge) carbon-coated copper grids (Science Services) and negatively stained with 2% aqueous uranyl acetate. Images of grids were recorded on a Phillips CM10 transmission electron microscope [Hendrick Dietz, Technical University of Munich (TU Munich)] operating at 100 kV and using a 4000-× 4000-pixel CCD camera at a resolution of 0.33 nm per pixel.

Single-Particle Analysis. The micrographs were processed using the Electron Micrograph Analysis 2 (EMAN2) (44) and System for Processing Image Data from Electron Microscopy (SPIDER) (45) software for particle picking, alignment, and classification. From 146 micrographs, 981 images of myosin cross-links or myosin filaments were manually picked and windowed with a window size of 300 × 300 pixels, large enough to comprise three myosin cross-links and three actin pseudopeptides. These images were used to determine the distance between adjacent cross-links. For further processing, including alignment, classification, and actin-polarity determination, the images were windowed again to 90 × 90 pixels, covering only a single myosin cross-link and used for structural analysis of the cross-links.

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