Toxoplasma gondii myosin A and its light chain: a fast, single-headed, plus-end-directed motor

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Successful host cell invasion is a prerequisite for survival of the obligate intracellular apicomplexan parasites and establishment of infection. Toxoplasma gondii penetrates host cells by an active process involving its own actomyosin system and which is distinct from induced phagocytosis. Toxoplasma gondii myosin A (TgMyoA) is presumed to achieve power gliding motion and host cell penetration by the capping of apically released adhesins towards the rear of the parasite. We report here an extensive biochemical characterization of the functional TgMyoA motor complex. TgMyoA is anchored at the plasma membrane and binds a novel type of myosin light chain (TgMLC1). Despite some unusual features, the kinetic and mechanical properties of TgMyoA are unexpectedly similar to those of fast skeletal muscle myosins. Microneedle−laser trap and sliding velocity assays established that TgMyoA moves in unitary steps of 5.3 nm with a velocity of 5.2 μm/s towards the plus end of actin filaments. TgMyoA is the first fast, single-headed myosin and fulfills all the requirements for power parasite gliding.

Keywords: invasion/myosin/myosin light chain/Toxoplasma gondii/transient kinetics

Introduction

The phylum of Apicomplexa consists mainly of medically and economically important pathogens that infect humans and animals. These obligate intracellular parasites actively penetrate their host in a few seconds by squeezing at 1−10 μm/s through a moving junction formed between the host cell and the parasite plasma membrane. Parasite replication then occurs safely within a non-fusogenic parasitophorous vacuole. The actomyosin-dependent gliding motility of Toxoplasma gondii tachyzoites and Plasmodium species sporozoites is an essential component of the invasion process (Sultan et al., 1997; Sibley et al., 1998). This unusual form of motility is not generated by the action of cilia or flagella but is probably driven by the redistribution of surface adhesin complexes towards the posterior pole of the parasite. The adhesive components of these complexes are presumed to establish a tight interaction with host cell receptors or extracellular matrix, while the cytoplasmic tail of the transmembrane proteins are anticipated to establish a direct or indirect connection with the actomyosin system (Dobrowolski and Sibley, 1997; Kappe et al., 1999). Time-lapse video microscopy revealed that T.gondii tachyzoites glide at a speed of 1−3 μm/s, based on a combination of three major modes of locomotion involving one or more myosin motor(s) (Hakansson et al., 1999). Five class XIV myosins have been identified so far in T.gondii (TgMyoA–E) (Heintzelman and Schwartzman, 1997; Hettmann et al., 2000; F.Delbac and D.Soldati, unpublished). While TgMyoB and TgMyoE are not expressed in tachyzoites, TgMyoC appears to play a role in parasite division (Delbac et al., 2001). The unique localization of TgMyoA at the plasma membrane makes it a primary candidate to propel the parasite into its host (Hettmann et al., 2000). Close homologues of TgMyoA are present in the genomes of Plasmodium spp. (Heintzelman and Schwartzman, 1997; Pinder et al., 1998; Hettmann et al., 2000; Matuschewski et al., 2001), Eimeria and Theileria. Our accumulated evidence indicated that TgMyoA is an essential gene and important for parasite gliding and host cell invasion. Indeed, among other approaches, a second copy of TgMyoA was introduced under the control of a tetracycline repressor system (Meiûner et al., 2001) and the endogenous copy was deleted by double homologous recombination. Unfortunately, due to an uncharacterized reversion mechanism, all survivors had lost the capacity to repress the expression of the inducible copy of TgMyoA by removal of anhydrotetracycline (data not shown). More recently, we have established a tet-transactivator (tTA) system for T.gondii and were able to disrupt the endogenous copy of TgMyoA successfully in the presence of a second copy controlled by a tTA-responsive promoter. Addition of anhydrotetracycline turns off expression of TgMyoA and prevents parasite gliding, host cell invasion and cell-to-cell spreading (M.Meissner, D.Schlüter and D.Soldati, manuscript submitted).

In a complementary approach to link TgMyoA to invasion mechanisms, we undertook a comprehensive dissection of its biochemical and biophysical properties. The motor(s) powering gliding is expected to be of a fast...
and did not co-fractionate on glycerol gradients (data not shown). The association of TgCaM with TgMyoA or TgMyoA could be ruled out as they did not co-purify (Figure 1C). Beside calmodulin, a calmodulin-like protein is shown). Raising specific antibodies against (TgCaM; previously described by Seeber et al., 1999). Association of TgCaM with TgMyoA or TgMyoA could be ruled out as they did not co-purify (Figure 1C) and did not co-fractionate on glycerol gradients (data not shown). Beside calmodulin, a calmodulin-like protein is present in the P.falciparum genome database as well as in several expressed sequence tag (EST) databases of other apicomplexan parasites including T.gondii. We cloned and characterized the gene coding for this T.gondii calmodulin-like protein and raised polyclonal antibodies against the first 75 amino acid residues of the protein fused to GST and expressed in Escherichia coli. The antisera detected a 31 kDa protein that, in contrast to TgCaM, co-fractionated with TgMyoA on the glycerol gradient (Figure 1A).

We conclude that, as predicted from the absence of a coiled-coil domain in the tail region, these experiments indicate that native TgMyoA is single headed (Figure 1A).

Further analysis by western blotting revealed that the 31 kDa protein co-purified with TgMyoA heavy chain in several native preparations but was absent when TgMyoA was purified under denaturing conditions or when native TgMyoA was purified (Figure 1B). Based on the characteristics defined in this study, we named this protein T.gondii myosin light chain 1 (TgMLC1). Consistent with the results obtained above, we also showed that the last 53 amino acid residues in the tail of TgMyoA were necessary and sufficient to bind TgMLC1 (Figure 1D). A stretch of His8 tag was introduced at the N-terminus of green fluorescent protein (GFP) in the pTGFP and pTGFPAtail constructs (Hettmann et al., 2000). HisGFP and HisGFPAtail proteins were expressed stably in T.gondii and purified from parasite lysates on Ni-NTA resin. TgMLC1 co-purified specifically with HisGFPAtail but not with HisGFP as detected by western blotting of the material eluted from the batch purification. Finally, additional data from another group allowed us to establish that the TgMyoA complex is at least heterotrimeric. It is composed of one TgMyoA heavy chain (93 kDa), one light chain (TgMLC1; 31 kDa) and a recently identified, 50 kDa myristoylated protein (TgMADP) which anchors TgMyoA at the plasma membrane (C.Beckers, International

Results

Purification of TgMyoA and identification of the myosin light chain associated with the motor complex

To determine the biochemical and biophysical properties of this motor, full-length TgMyoA and TgMyoA (a mutant lacking 53 C-terminal residues) were purified from recombinant parasites. The apparent molecular mass of the two proteins was determined by glycerol gradient centrifugation (Figure 1A). As expected, TgMyoAAtail fractionated close to its molecular size of ~100 kDa. In contrast, TgMyoA had an apparent size of 160 kDa, clearly smaller than a dimer, but nevertheless bigger than was predicted by the 53 residues difference between the two proteins. Despite the lack of clearly identifiable binding site in the neck/tail of TgMyoA, we searched for the presence of a light chain. The neck domains of many unconventional myosins bind calmodulin, therefore we raised specific antibodies against T.gondii calmodulin (TgCaM; previously described by Seeber et al., 1999). Association of TgCaM with TgMyoA or TgMyoA could be ruled out as they did not co-purify (Figure 1C) and did not co-fractionate on glycerol gradients (data not shown). Beside calmodulin, a calmodulin-like protein is present in the P.falciparum genome database as well as in several expressed sequence tag (EST) databases of other apicomplexan parasites including T.gondii. We cloned and characterized the gene coding for this T.gondii calmodulin-like protein and raised polyclonal antibodies against the first 75 amino acid residues of the protein fused to GST and expressed in Escherichia coli. The antisera detected a 31 kDa protein that, in contrast to TgCaM, co-fractionated with TgMyoA on the glycerol gradient (Figure 1A).

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of the parasite penetrating the host cell (Figure 2A). Less
than 10 min later, TgMLC1 was still distributed at the
moving junction and in the part of the parasites. (C) At 24 h post-invasion, a double immunofluorescence with
anti-MLC1 and anti-SAG1 under permeabilized conditions showed co-localization of both proteins. Scale bar: 1 µm.

TgMLC1 co-localized with TgMyoA at the parasite plasma membrane
As anticipated for a TgMyoA-associated protein, TgMLC1 localized at the parasite plasma membrane under static conditions (intracellular parasites) (Figure 2C). In addition, during host cell penetration, TgMLC1 was slightly concentrated at the moving junction and in the part of the parasite penetrating the host cell (Figure 2A). Less than 10 min later, TgMLC1 was still distributed at the parasite periphery but also revealed a circle at the posterior end, delineating an open ‘appendage’ at the parasite posterior extremity (Figure 2B). This structure, visible only shortly after invasion, is likely to correspond to the transient posterior invagination where multi-lamellar vesicles are released to form the intravacuolar network (Sibley et al. 1995).

TgMyoA binds a novel type of light chain and both proteins are restrictively conserved throughout the Apicomplexa
TgMLC1 comprises 213 amino acids and appears to be composed of four degenerate EF hand domains (Figure 3A). Structural homology searches indicated that only the third one (residues 149–179) fits the consensus sequence well; the first one is relatively conserved but the other two carry multiple deletions. Very closely related proteins of similar sizes are present in the genome of several Apicomplexa and most probably correspond to the light chain of MyoA homologues. The N-terminal 60 residues are unique, not present in calmodulin or other myosin light chains and highly conserved in the Apicomplexa. TgMyoAA\_tail was shown to be cytosolic, and the determinant of plasma membrane localization of TgMyoA mapped within its last 22 residues and was strictly dependent on an RR motif (Hettmann et al., 2000) (see Figure 3B, bar and double arrowhead). Interestingly, deletion of the tail prevents co-purification of TgMLC1 and TgMADP. Close examination of the last 22 residues reveals the presence of a very degenerate IQ motif (QXXXR, Figure 3B). The precise topology of the TgMyoA–TgMLC1–TgMADP complex remains to be determined.

Transient kinetics of TgMyoA ATPase
The heterotrimeric TgMyoA complex could only be purified in very small amounts, which were insufficient for kinetic analysis using stopped-flow methods but were ample for use in the flash photolysis system developed for limited quantities of myosins (Weiss et al., 2000). In contrast, transient kinetic data for monomeric TgMyoA\_tail were acquired by both stopped-flow and flash photolysis methods. The maximum velocity at which myosins move over actin is normally limited by the net rate of cross-bridge detachment at the end of the power stroke (Siemankowski and White, 1984; Weiss et al., 2001). This detachment rate can be limited by the rates of both ADP release and ATP binding, measurements of which can therefore be diagnostic of maximum gliding velocity. The rate constant of the ATP-induced dissociation of both full-length TgMyoA and TgMyoA\_tail from actin was determined by the flash photolysis method as shown in Figure 4A. A known concentration of ATP was released from 0.5 mM caged ATP in a sample 0.5 µM actin and 1 µM TgMyoA and the dissociation of the complex monitored by the decrease of the light scattering signal. A single exponential fit to the light scattering trace gave the k\_obs of the reaction (Figure 4A). The experiment was repeated at various ATP concentrations and a plot of k\_obs versus [ATP] was linear over the range of concentrations used. The slope of the linear regression defines the apparent second-order rate constant K\_d = 0.28 and 0.46/µM/s for full-length TgMyoA and TgMyoA\_tail, respectively (Figure 4B).

The affinity of ADP for acto-TgMyoA was measured from the ADP inhibition of the ATP dissociation experiment. A constant 50 µM ATP was photoreleased in the presence of increasing amounts of ADP (0–2 mM). The value of k\_obs decreased as the ADP concentration increased, and the resulting data were described by a hyperbola (Scheme I, see Table I footnotes), suggesting that ADP binding and dissociation is in rapid equilibrium with acto-TgMyoA preceding ATP-induced dissociation of the complex (Scheme II). The analysis defined the ADP affinity, K\_AD, as 840 and 720 µM for TgMyoA and TgMyoA\_tail, respectively (Figure 4C). The flash photo-
Fig. 3. Sequence alignments of apicomplexan myosin light and heavy chains. (A) Myosin light chains from *P. falciparum*, *Eimeria tenella*, *Neospora caninum* and *T. gondii*. TgMLC1 accession No. AY048862. The position of the four proposed degenerate EF hand domains is indicated by a bar. (B) Alignment of the C-terminus of class XIV myosin heavy chains from *T. gondii*, *Plasmodium* spp., *E. tenella* and *Theileria parva*. (C) Alignment of myosin sequences around the converter domain and the lever arm helix of selected myosins of different classes that have been characterized biochemically, except PfMyoA. Myosin VI is the only minus-end-directed myosin characterized so far. The alignment starts at position 650 of *D. discoideum* myosin II and ends at residue 821. The arrowhead indicates where the TgMyoAIL tail was truncated. The structural domains are indicated at the top of the alignment, as described by Geeves and Holmes (1999). The asterisk indicates two cysteines (SH1, SH2) conserved in class II myosins; the hash sign indicates three glycines conserved among most myosin classes. The first glycine is the proposed ‘pivot point’ corresponding to Gly699 of GgFSk (Kinose et al., 1996), and is absolutely conserved except in a plant myosin (HaMyoK3) and a *Caenorhabditis elegans* class XII myosin. Conservation between class XIV myosins and others is highest upstream of and including the actin-binding domain. Residues conserved in at least six of eight sequences are in red; residues conserved in at least four of the five non-class XIV myosins are in green; and the C-terminus highly conserved among class XIV myosins is in blue. Residues conserved in at least six of eight sequences are in red; residues conserved in at least four of the five non-class XIV myosins are in green; and the C-terminus highly conserved among class XIV myosins is in blue. Myosin VI-specific insertion at the end of the converter domain is cut out of the alignment and presented below in pink. *Dictyostelium discoideum* myosin II (DdII, accession No. P08799); *Gallus gallus* (chicken) fast skeletal myosin II (GgFsk, P13538); *G. gallus* brush border myosin I (GgBb, U04049); *G. gallus* p190 myosin V (Ggp190V, Z17118); *Sus scrofa domesticus* myosin VI (SsVI, A54818); *P. falciparum* myosin A (PFMyoA, AAD21242); *T. gondii* myosin D (TgMyoD, AFI05118); *T. gondii* myosin A (TgMyoA, AAC7724).

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MOPS, 5 mM MgCl$_2$ and 10 mM fresh DTT at pH 7.0 and 22°C. Lysis experiments showed that the values of both $K_A$ and $K_D$ were similar for full-length TgMyoA and TgMyoAΔtail and demonstrate that the truncation of the tail by 53 residues does not alter the kinetic properties of the motor domain of TgMyoA.

A more complete kinetic characterization by stopped flow was then undertaken with the more readily available TgMyoAΔtail. The ATP-induced dissociation and the ADP inhibition of the dissociation were measured using 400 nM acto-TgMyoA complex. Unlike any other myosin so far examined, TgMyoA (and TgMyoAΔtail) did not quench the fluorescence of a pyrene label covalently attached to actin. Therefore, dissociation of the complex was measured by light scattering. The results are given in Table I and although the stopped-flow measurements resulted in a higher value of $K_A$ and a lower value of $K_D$ than flash photolysis, the values are of the same order for both methods. The data confirm that TgMyoA is dissociated quickly from actin by ATP and that the TgMyoA·actin complex has a low affinity for ADP, properties that TgMyoA shares with fast muscle myosins.

The rate of ATP binding to TgMyoAΔtail was determined by measuring the intrinsic tryptophan fluorescence change which occurs on ATP binding. A 3% decrease in fluorescence was observed compared with the usual fluorescence increase obtained for other myosins, probably due to the absence of the tryptophan normally thought to be responsible (Batra and Manstein, 1999). The slope of the linear fit to a plot of $k_{obs}$ versus [ATP] gives $K_A = 0.52\mu$m/s (Table I). This was repeated using the fluorescent analogues mantATP and mantADP and gave similar second-order binding rate constants. For ADP, the dissociation rate constant was estimated by displacing bound mantADP with a large excess of ATP, resulting in a value of $k_{AD} = 0.58$/s. The affinity of TgMyoA for mantADP was estimated as 2 µM from the relationship $K_f = K_A + 2k_{AD}/k_{AD}$. The affinity of actin for TgMyoA was measured in a competition experiment with rabbit myosin S1. Under the conditions used, TgMyoA had a 2- to 3-fold lower affinity for actin than S1. Under physiological conditions, the dissociation equilibrium constant ($K_A$) of S1 for actin is 30 nM, suggesting a value of 60–90 nM for TgMyoA·actin. To determine ATP turnover activity of TgMyoAΔtail, 50 nM TgMyoAΔtail with 50 or 200 µM ATP were quenched at various time points. The analysis of [ATP]/[ADP] ratios with an HPLC system gave an ATP turnover rate of 0.07/s in the absence and 1.5/s in the presence of 20 µM actin. A 20-fold activation in the presence of actin is typical of fast myosins.

**Determination of TgMyoA velocity and step size**

To complement this analysis, we directly determined some of the functional properties of this motor. The sliding velocity of actin filaments was measured in a standard *in vitro* motility assay (Kron and Spudich, 1986). We measured an average velocity of 5.2 µm/s for TgMyoA (Figure 5A). TgMyoAΔtail showed no motility under the same conditions, although the protein displayed ATP-dependent binding to actin. This lack of motility is probably due to a functional defect caused by deletion of the last 53 C-terminal residues, which hampers association with TgMLC1. The sequences of class XIV myosins diverge from that of other myosins, starting at a position corresponding to the SH1–SH2 region, just downstream of the 50k–20k domain junction (Figure 5C). Thus, TgMyoA...
displays little sequence similarity to other types of myosin in regions that normally form the converter and lever arm. The recent finding that myosin VI, exhibiting an unusual converter domain, is a minus-end-directed motor (Wells et al., 1999) prompted us to determine the directionality of TgMyoA. For this purpose, the in vitro motility assay was performed with actin filaments labelled with Oregon green at their plus (barbed) ends. To block actin polymerization at the plus end of the labelled tips, they were generated in the presence of gelsolin (Figure 5B). TgMyoA moved these polarity-marked filaments so that the labelled end was at the back of the moving filament, demonstrating that TgMyoA, like myosin II, moves towards the plus end of F-actin.

Furthermore, we employed a combined microneedle–laser trap transducer (Ruff et al., 2001) and directly measured the step displacements generated by single TgMyoA molecules. In agreement with our kinetic analysis, TgMyoA interacted transiently with actin filaments. A histogram analysis of 598 such interactions revealed an average step displacement of ~5.3 nm (Figure 6), comparable with the step size reported for myosin II (Veigel et al., 1999). In addition, these measurements confirm at the single molecule level that TgMyoA is a non-processive motor. Taken together, the sliding velocity and step size measurements of full-length TgMyoA are in perfect agreement with, and thus validate, the kinetic values.

**Discussion**

The catalytic domains and neck regions are remarkably conserved between myosin classes. Even in myosin VI, which displays motility towards the pointed end of actin filaments, there is clear structural conservation throughout most of the catalytic domain. TgMyoA shows some unusual features such as the lack of a glycine in the SH1 helix, a degenerate IQ motif and a low degree of sequence similarity with the converter region of conventional myosins. However, these differences are not sufficient to infer that TgMyoA lacks classical converter and light chain-binding domains that act as a conformational amplifier. Generally, the converter domains of myosins are not well conserved between classes outside a few critical residues. Functional conservation is suggested by the fact that most of the conserved hydrophobic residues in TgMyoA are found buried between the relay loop and the converter domain, where they play an essential role for the transmission of information between the nucleotide-binding site, the actin-binding site and the lever arm (Geeves and Holmes, 1999). This study established that TgMyoA, despite some unusual features, fulfils the
biochemical requirements for a fast motor involved in gliding (King, 1988; Hakansson et al., 1999). Like fast muscle myosins, TgMyoA has a short ‘duty cycle’, is thus non-processive and most probably operates in the context of large motor arrays. The fast release of ADP suggests that for this myosin, like other fast myosins, the rate-limiting step is either the phosphate release step or a preceding step such as the ATP cleavage. At an ATP concentration of 1 mM, we observed an average lifetime of the events of ~350 ms. This event duration is similar to the one we previously observed under identical conditions for skeletal muscle myosin (Ruff et al., 2001) and is also in good agreement with our kinetic analysis.

We provide here biochemical evidence that eliminates the possibility that TgCaM serves as light chain for this myosin of the class XIV. Furthermore, both the solubility and localization of TgCaM in the parasites are not compatible with an association with TgMyoA (data not shown). Instead, we identified a novel type of light chain bound to the last 53 amino acids at the C-terminus of TgMyoA heavy chain. TgMyoA associated with TgMLC1 and TgMADP is distributed homogeneously beneath the parasite’s plasma membrane (Hettmann et al., 2000), ready for a consolidated action. In accordance with the ‘capping model’ for invasion (Sibley et al., 1998), the directionality of TgMyoA implies that the actin filaments ought to be oriented with their plus ends toward the posterior pole. Surprisingly, both fluorescent phalloidin staining and sedimentation of crude cytoskeleton failed to detect actin filaments in parasites (Dobrowolski et al., 1997). This indicates that filaments might be very short and possibly undergo high turnover due to the abundant presence of actin-depolymerizing factors (Allen et al., 1997), and polymerize only in response to signalling generated by host cell contact at the time of invasion. Recently, treatment of parasites with the actin filament-stabilizing drug jasplakinolide was shown to induce an acrosomal process. Although actin filaments were clearly visible under these non-physiological conditions, their polarity could not be resolved (Shaw and Tilney, 1999).

The elaborate gliding motions of apicomplexan parasites accompanying host cell penetration allow entry significantly more quickly than by passive uptake via phagocytosis by host cells (Morisaki et al., 1995). This specific adaptation may serve as a selective escape mechanism, leading to the formation of a unique vacuole unable to fuse with endocytic compartments (Joiner et al., 1990). To accomplish such a fast process, one might speculate that the apicomplexans have learned to exploit the actin cytoskeleton fully, using a combination of myosin-powered motion together with the force generated directly by actin polymerization (reviewed in Theriot, 2000).

Materials and methods

Parasites strains, cell culture and reagents

Transgenic T. gondii parasites expressing the constructs pS-TgMyoA-tail and pS-TgMyoA were used as a source of recombinant proteins (Hettmann et al., 2000). Toxoplasma gondii tachyzoites were grown in Vero cells (African green monkey kidney cells) in tissue culture flasks, maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 25 μg/ml gentamicin. Rabbit polyclonal antibodies were raised against a bacterially
expressed GST fusion of 75 amino acids corresponding to the N-terminus of TgMLC1.

**Protein purification**

TgMyoAΔtail was purified as described previously (Hettmann et al., 2000). Full-length His-tagged TgMyoA was purified similarly with the following modifications. The parasite pellet (10^10 parasites) was lysed by sonication in a buffer with detergent [2% Triton X-100, 4 mM ATP, 2 mM MgCl_2, 0.5 mM dithiothreitol (DTT)], pH 7.6 and 1× Complete protease inhibitor cocktail (Roche). The suspension was clarified by ultracentrifugation at 60,000 rpm and the supernatant was applied to an Ni-NTA resin column and eluted according to the manufacturer (The Qiagen protocol, 2011). For the purification under denaturing conditions, the cells were lysed in the presence of 6 M guanidinium chloride and the clarified lysate was dialysed in the presence of 8 M urea before application on the column. Protein concentration was determined by Bradford assay (Bio-Rad). SDS–PAGE was performed by standard methods. Average yields of 30 μg of TgMyoA and 250 μg of TgMyoAΔtail were obtained per gram of parasites. Active forms of TgMyoAΔtail and TgMyoA were purified further by co-purification with F-actin and release in the presence of ATP (Anson, 1929), Myosin subfragment 1 (S1) was prepared by chemotryptic digestion of rabbit myosin as described by Weeds and Taylor (1975). The molar concentration was determined from the extinction coefficient at 280 nm (ε^280 = 7.9/cm) and a molecular weight of 115 kDa. Rabbit skeletal actin was purified by the method of Spudich and Watt (1971). Its molar concentration was determined by absorbance at 280 nm (ε^280 = 1.104/cm) and a molecular weight of 42 kDa (West et al., 1967). The preparation of pyrene-labelled actin was described previously (Criddele et al., 1985). To prepare phallolidin-stabilized F-actin (RhPh/actin), a stock solution of 10 μM F-actin and 10 μM phallolidin was incubated in experimental buffer overnight (Kurzawa and Geeves, 1996). Unless noted otherwise, all experiments with myosin S1 were performed in experimental buffer containing 100 mM KCl, 20 mM MOPS and 5 mM MgCl_2 at pH 7.0 and 5 mM MgCl_2 at pH 7.0). Samples in standard buffer (except 30 mM KCl) were quenched at various times by the addition of 2 mM ATP containing 2 mM ATP. For manual analyses, tapes were replaced from the VCR to the Argus 20 and individual filaments tracked frame-by-frame using a mouse. Velocity was calculated from the positional change and time code recorded on the VCR. Experiments with TgMyoA and TgMyoAΔtail were performed with two freshly made protein preparations each. Chicken fast skeletal muscle HMM (8.5 ± 1.5 μm/s) and Dictyostelium discoideum myosin II (2.4 ± 0.3 μm/s) were used as positive controls and >100 filaments were analysed in each case. For protein purification, see Ruppel et al. (1994) and Kron and Spudich (1986).

**Glycerol gradient centrifugation**

The centrifugation of 12 ml of glycerol density gradients was performed as described previously (Mitchell et al., 1997). The 10 and 30% glycerol solutions contained 50 mM NaH2PO4 and 300 mM NaCl pH 7.6. TgMyoA purified through the Ni-NTA column was loaded onto gradients after ultracentrifugation and overnight dialysis at 4°C. Filaments were imaged with a 1.3 NA 40× oil immersion objective with a 1.3 × 100 μm/° field of view. Optical sections were imaged with a Zeiss 1.4 NA 100× oil immersion lens by a Zeiss Axioskop with a cooled charge-coupled device (CCD) camera (Hamamatsu Photonics, Argus 20) and time-date generator, by an S-VHS VCR (JVC BRS800-E). After focusing and observation of the actin filaments in rigor, motion was initiated by infusing 100 μL of buffer containing 2 mM ATP. For manual analyses, tapes were replaced from the VCR to the Argus 20 and individual filaments tracked frame-by-frame using a mouse. Velocity was calculated from the positional change and time code recorded on the VCR. Experiments with TgMyoA and TgMyoAΔtail were performed with two freshly made protein preparations each. Chicken fast skeletal muscle HMM (8.5 ± 1.5 μm/s) and Dictyostelium discoideum myosin II (2.4 ± 0.3 μm/s) were used as positive controls and >100 filaments were analysed in each case. For protein purification, see Ruppel et al. (1994) and Kron and Spudich (1986).

**Preparation of polarity-labelled actin filament**

Oregon green-labelled G-actin was mixed with gelsolin at a ratio of 33:1 and incubated on ice for 20 min. Adding 100 mM KCl at room temperature started actin polymerization. The resulting F-actin seeds were stabilized with phallolidin. Elongation of these seeds is restricted to the pointed end, because their barbed ends are blocked by gelsolin. For elongation, a 10-fold excess of unlabelled G-actin was added in Tris buffer containing 100 mM KCl, and the reaction was allowed to proceed for 15 min at room temperature. Finally, the polarity-labelled F-actin was diluted and the elongated part of the filament was stabilized with rhodamine–phallolidin. An Olympus IX70 inverted microscope with a Zeiss 1.4 NA 100× oil immersion objective was used to image the filaments by epifluorescence. A Polychrom II monochromator (Till Photonics, Munich, Germany) in combination with a dual-band filter set (Chroma, Brattleborough, USA) was used for excitation at 492 and 554 nm. Filaments were imaged using a Till Imago CCD camera, and TillVision was used for wavelength selection and image acquisition.

**Step size measurements**

Single actomyosin interactions were recorded with a combined microneedle–laser trap system as described previously (Ruff et al., 2001). This setup is similar to the double-laser trap system utilizing a two-lens geometry (Finer et al., 1994), except that we replaced one of the trapped beads with a fine glass microneedle (stiffness 0.01-0.03 pN/nm, time constant ~1 ms). A magnified brightfield image of the needle projected onto a quadrant photo-diode (Hamamatsu, S1557) allows position detection with subnanometer resolution and a bandwidth of >8 kHz. Signals were low-pass filtered (1 kHz), sampled at 5 kHz with a personal computer and stored on a hard disk to allow off-line analysis. Single actomyosin interactions in vitro assays were carried out in standard motility buffer (25 mM imidazole pH 7.4, 25 mM KCl, 1 mM EGTA, 4 mM MgCl_2, 1 μM ATP, 10 mM DTT, 100 μM glucose oxidase, 18 μg/mL catalase and 3 mg/ml glyceraldehyde 3-phosphate). To record from a single TgMyoA motor, we constructed a recording chamber from two coverglasses that were separated by a 1 mm Teflon spacer. The lower coverglass was coated with a 0.1% nitrocellulose solution (Fullam Latham, NY) in amyl acetate containing 2.5 μm diameter silica beads (Bangs Laboratories, Fishers, IN) which served as pedestals. TgMyoA (1.5 μg/ml) was directly adsorbed onto nitrocellulose-coated surface by incubating for 15 min. We inserted a microneedle from the open side into the recording chamber and, using NEM-myosin as glue, attached a single actin filament at one end to the tip of the microneedle and at the other end to a 1 μm latex bead captured in the laser trap. The entire arrangement of microneedle, actin filament and latex bead was pulled taut, lowered carefully onto the myosin-coated bead and the ensuing actomyosin interactions were recorded. To determine the step size, we followed the strategy of Molloy et al. (1995). We identified single actomyosin interactions by a reduction of the thermal fluctuation of the microneedle sensor during binding to myosin. From the average position of a large number of such events, we calculated a histogram and determined the step size from the shift of the mean position of the distribution.
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References


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