

# Intracellular localization and dynamics of myosin-II and myosin-IC in live *Acanthamoeba* by transient transfection of EGFP fusion proteins

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## Summary

We developed a reliable method for transient transfection of *Acanthamoeba* using Superfect (Qiagen) and a vector with the *Acanthamoeba* ubiquitin promoter and enhanced green fluorescent protein (EGFP) as the reporter gene. The transfection efficiency was 3% for profilin-I-EGFP and EGFP-myosin-II tail, and less than 0.5% for larger constructs such as full length myosin-II or myosin-IC. Profilin-I-EGFP was distributed throughout the cytoplasm as observed previously with rhodamine-labeled profilin, while EGFP alone accumulated in the nucleus. EGFP fused to full length myosin-II or to the C-terminal 256 residues of the myosin-II tail concentrated in fluorescent spots similar to thick filaments and minifilaments identified previously in fixed cells with fluorescent antibodies. Thick filaments were located in the dorsal cytoplasm and along the lateral margins of the back half of the cell. Thick

filaments formed behind the leading edge and moved continuously towards the rear of the cell, where they disassembled. If phosphorylation of the myosin-II heavy chain was prevented by mutation of all three phosphorylated serines to alanine, thick filaments of unphosphorylated myosin-II accumulated around vesicles of various sizes. EGFP-myosin-IC was spread throughout the cytoplasm but concentrated transiently around contractile vacuoles and macropinocytosis cups providing that the construct included both the head and a tail with the SH3 domain.

Movie available online

Key words: Myosin, Motility, Microscopy, Green fluorescent protein, Transfection

## Introduction

The enzymology, assembly and regulation of *Acanthamoeba* myosin-I and myosin-II have been studied in detail (Sinard and Pollard, 1989; Brzeska et al., 1992; Ostap and Pollard, 1996). The three known myosin-I isoforms have distinct distributions in fixed cells judging from fluorescent antibody and immunogold methods (Baines and Korn, 1990; Baines et al., 1995). Only myosin-IC concentrates around contractile vacuoles and antibodies specific for myosin-IC inhibit contractile vacuole function in live cells (Doberstein et al., 1993). Fluorescent antibody staining of fixed cells showed that myosin-II forms particles of two sizes (Yonemura and Pollard, 1992) corresponding to mini-filaments of eight molecules and thick filaments formed by lateral association of mini-filaments (Sinard and Pollard, 1989).

Myosin dynamics have been documented in animal cells (Wei and Adelstein, 2000; Buss et al., 2001; Tang and Ostap, 2001), *Dictyostelium* (Moores et al., 1996; Zang and Spudich, 1998; Yumura, 2001; Neujahr et al., 1997) and yeast (Bezanilla et al., 2000; Lee et al., 2000) by expressing myosin fusion proteins tagged with green fluorescent protein (GFP). Such GFP fusion proteins can take the place of wild-type *Dictyostelium* myosin-II (Moores et al., 1996), yeast myosin-II (Bezanilla et al., 2000) and yeast myosin-I (Lee et al., 2000).

Analysis of the cellular dynamics of the biochemically well-characterized *Acanthamoeba* myosins has been hampered by the lack of a robust transfection system. Hu and Henney (Hu and Henney, 1997) had some success transfecting *Acanthamoeba* by electroporation with a CAT (chloramphenicol acetyltransferase) reporter gene under the control of the amoeba ubiquitin promoter. We have improved the transfection method for *Acanthamoeba*, so that transient expression of a variety of GFP-fusion proteins is now routine.

We find that myosin-II assembles into particles of two sizes, interpreted as mini-filaments and thick filaments. Thick filaments form anteriorly and laterally and then move to the rear of the cells where they disassemble. In cells also expressing full length myosin-II, only the C-terminal 256 residues of the myosin-II tail are required for the GFP-fusion protein to go through this cycle of assembly and disassembly. Mutation of the inhibitory phosphorylation sites in the tail piece of myosin-II to alanine resulted in abnormal accumulation of the construct around vacuoles in the cell. Myosin-IC accumulates around contractile vacuoles seconds before they discharge, as well as around macropinocytosis cups just before they pinch off. Localization to these two sites requires both the head of the myosin and most of the tail, including the SH3 domain.

**Table 1. Primer sequences for PCR amplification of the primers and genes used to make expression vector in the present paper**

Gene	Direction	Sequence	
Ubiquitin promoter	Sense	TAAGAGCTC*TAGCACAAACTCGGCCAC	
	Antisense	TAACCATGG†GTCGACGTACGTCTGCCACCA	
EGFP	N-terminal	Sense	TTACCATGG†TGAGCAAGGGCGAGCTG
		Antisense	TTAACTAGT§CTTGTACAGCTCGTCCATGCC
	C-terminal	Sense	TTAACTAGT§ATGGTGAGCAAGGGCGAGCTG
		Antisense	TTATCTAGA‡TACTTGTACAGCTCGTCCATGCC
Profilin I	Sense	TTACCATGG†AACCCTCTACCACCACCC	
	Antisense	TTAACTAGT§AAAGCCCTGACCGATGAGGTAG	
Myosin II	Tail	Sense	TAAACTAGT§GTGAAGCGCCTCGAGGGCGAG
		Antisense	TAACTCTAGA‡TTATTTTATGGGTGTATAAAAG
	Full length	Sense	TTAACTAGT§ATGGCCGCCAGCGCAGGAGG
		Antisense	TAACTCTAGA‡TTATTTTATGGGTGTATAAAAG
Phosphorylation site mutated full length	Sense	TTAACTAGT§ATGGCCGCCAGCGCAGGAGG	
	Antisense	TCTAGA‡TTACTCCTCGGCGCGGGCGGAGCCGGCCCTCACGGCGGCACCGCGGGCCG□GGCGCCGCGGGTGG□	
Myosin IC	Full length	Sense	TAAACTAGT§ATGGCGTATACGAGCAAGCATGGC
		Antisense	TAACTCTAGA‡TTACATGCCGGGGGAGGAGGACC
pUbGMICb	Antisense	TAACTCTAGA‡TTACTTGTGGCGTAGGAGAACAC	
pUbGMICc	Antisense	TAACTCTAGA‡TTAGAGGATCTGGTCTTGTAGGA	
pUbGMICd	Antisense	TAACTCTAGA‡TTAGGGTCCGGGCGCGGACGCGGC	
pUbGMICe	Antisense	TAACTCTAGA‡TTAGATGAGTTCGACGTAGGACGC	
Tail only	Sense	TTAACTAGT§AAGAAGGAGCGTCTGCCTGTCC	

Sequences underlined are restriction enzyme sites for digestion and ligation into the vector (\**Sac*I site, †*Nco*I site, ‡*Xba*I site and §*Spe*I site). Sequences in the box are changed sequences to mutate phosphorylation site of myosin II tail. Sense primer of full length myosin IC was also used to amplify the four truncated mutants and antisense primer of full length for tail only construct.

## Materials and Methods

### Expression vector construction

The *Acanthamoeba* expression vector was modified from the Promega (USA) pGL3-Promoter vector (Fig. 1). *Sac*I and *Nco*I restriction sites were added to the ends of the *Acanthamoeba* ubiquitin promoter Ubp (kindly provided by H. Henney, Jr) to replace the SV40 promoter of pGL3 (Table 1). The human codon-optimized enhanced green fluorescent protein (EGFP) was substituted for luciferase as the reporter protein. EGFP was amplified with flanking *Nco*I, *Spe*I or *Xba*I restriction sites by PCR. The coding sequence of *Acanthamoeba* profilin-I was amplified by PCR from genomic DNA and inserted between Ubp and EGFP to make construct pUbPG. Plasmid DNA was prepared with either a Maxi prep kit or Endofree Maxi prep kit (Qiagen, USA). A cDNA encoding the C-terminal 256 amino acids and 78 bp of 3' UTR of the *Acanthamoeba* myosin-II heavy chain was amplified from a cDNA by PCR and inserted after EGFP to make plasmid pUbGMII. Full length myosin-II heavy chain cDNA was amplified by PCR and ligated into *Spe*I and *Xba*I sites after EGFP to make the plasmid pUbGMII. All three phosphorylation sites in the myosin-II tail piece (S1489, S1494 and S1499) were replaced with alanine by changing the first base T of the serine codons to G for alanine for the plasmid pUbGMII. Myosin-IC cDNA was amplified from a full length myosin-IC cDNA provided by E. D. Korn of NIH. Myosin-IC PCR products were inserted after EGFP to make plasmids for expression of EGFP fused to full length myosin-IC (pUbGMICf), myosin-IC

tail (pUbGMICt), myosin-IC truncated after the SH3 domain (pUbGMICs), myosin-IC truncated after GPA-1 (pUbGMICg), myosin-IC truncated after the basic region (pUbGMICb) or myosin-IC lacking the tail (pUbGMICh).

### Transfection

*Acanthamoeba castellanii* Neff strain grown to mid log phase in a bubbler at room temperature were washed twice with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub> per liter of water, pH 7.4) and resuspended in PYG culture medium (20 g Proteose Peptone, 1 g yeast extract, 4 mM MgSO<sub>4</sub>, 0.4 mM CaCl<sub>2</sub>, 3.4 mM sodium citrate, 0.05 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.5 M KH<sub>2</sub>PO<sub>4</sub>, pH 6.5, in 950 ml deionized water and autoclaved followed by addition of 50 ml of sterile-filtered 2 M glucose). Cells were cultured overnight at 25°C in a 6-well culture plate with 4×10<sup>5</sup> cells per well in 3 ml of culture medium. Four micrograms of plasmid DNA in 100 μl of amoeba culture medium were mixed with 20 μl of Superfect (Qiagen), incubated for 10 minutes at room temperature and then diluted with 600 μl of culture medium. Adherent amoeba in a 6-well plastic culture plate were washed once with room temperature PBS. After removing most of PBS, DNA-Superfect mixture was added drop-wise to the cells. Cells were incubated at 25°C for 3 hours to allow uptake of DNA-Superfect complexes. Cells were washed once with PBS, resuspended in 3 ml of fresh growth medium and incubated at 25°C for 24–48 hours. Expression of EGFP was checked by fluorescence microscopy.

### Flow cytometry and cell sorting

Cells were washed twice and resuspended in 2 ml of PBS. EGFP expression of  $1 \times 10^6$  cells was measured with a CellQuest 3.2 FACScan (Becton-Dickinson). For microscopic examination and immunoblots, transfected cells expressing EGFP were isolated by fluorescence-activated cell sorting using a Facstar (Becton-Dickinson) with a single laser.

### Microscopy

Sorted amoeba expressing green fluorescent protein were allowed to adhere to a glass-bottomed microwell during incubation for 1-2 hours at room temperature in PBS. Alternatively, transfected amoebae were flattened with an agar overlay (Yumura et al., 1984). Excess PBS was absorbed from the edge of the agar slice with filter paper until the cells flattened appropriately. To increase the frequency of contractile vacuole discharge, cells were washed briefly in PBS, 1:1 PBS:water, and incubated in water for 10 minutes at room temperature. Live cells were imaged with an Olympus IX70 fluorescent microscope with 100 $\times$  objective, cooled CCD camera and motorized Z-axis stage. Images were processed with Photoshop 5.5.

### Immunoblots

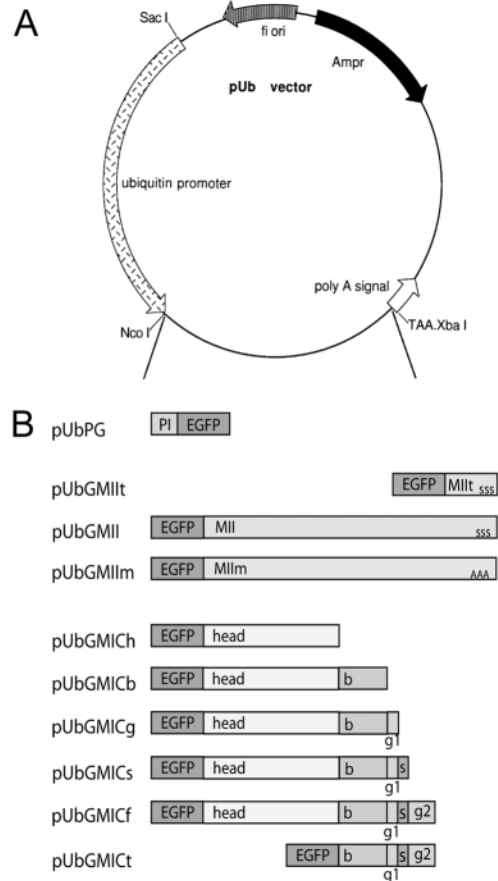
One thousand amoeba sorted by their green fluorescence were boiled in SDS-PAGE sample buffer, electrophoresed on a 15% acrylamide gel and blotted on a nylon membrane. Blotted proteins were detected with the following antibodies: a mixture of mouse monoclonal antibodies that react with *Acanthamoeba* profilin-I and profilin-II (Kaiser and Pollard, 1996); a mixture of 13 monoclonal antibodies specific for the C-terminal region of the *Acanthamoeba* myosin-II tail (Kiehart et al., 1984); actin monoclonal antibody c4d6 (Lessard, 1988); and mouse monoclonal anti-GFP (Zymed, San Francisco, CA). To reduce non-specific reactions, the anti-GFP antibody was preabsorbed with untransfected cells prepared by methanol fixation. Bound antibodies were detected by chemiluminescence.

## Results

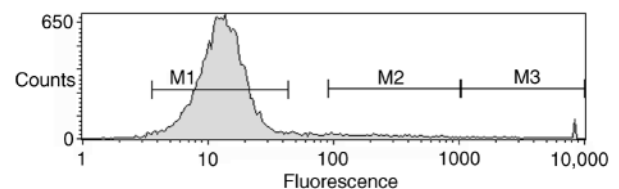
### Transient transfection

The transfection efficiency of ten EGFP constructs (Fig. 1B) depended on the size of the plasmid. About 3% of amoebas expressed EGFP (Fig. 2) when transfected with 20  $\mu$ l of Superfect and 4  $\mu$ g of the smallest plasmids, either pUbG (a 5672 bp plasmid for expression of free EGFP), pUbPG (a 6061 bp plasmid for expression of the profilin-I-EGFP fusion protein) or pUbGMIIIt (a 6448 bp plasmid for expression of the EGFP-myosin-II tail fusion protein). Transfection was less efficient with larger plasmids (Fig. 1B) such as pUbGMIIIf (a 10,465 bp plasmid for expression of EGFP fused to full length myosin-II). All combinations of 1 to 8  $\mu$ g of DNA and 4 to 40  $\mu$ l of Superfect yielded a maximum of 0.5% of cells expressing EGFP with these large plasmids.

We used profilin-I-EGFP to optimize the transfection conditions. Superfect was much more efficient than electroporation or any other method that we tested. Fresh amoeba and fresh DNA gave the best results. More cells were transfected with 4  $\mu$ g of plasmid DNA in Superfect than Hu and Henney (Hu and Henney, 1997) reported with electroporation of 100  $\mu$ g of DNA. In our hands electroporation produced very few transfected cells. The codon usage of EGFP is closer to that of *Acanthamoeba* than GFP, but modifying three EGFP codons that are rarely, if ever, used by *Acanthamoeba* might improve further the performance of the vector.

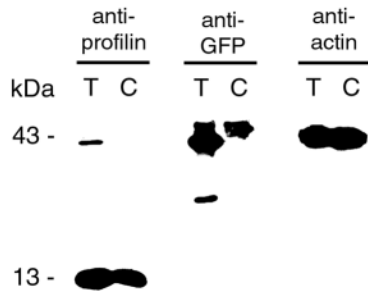


**Fig. 1.** *Acanthamoeba* expression vectors. (A) pUb vector has an *Acanthamoeba* ubiquitin promoter (Ubp). DNAs for EGFP fusion proteins were inserted at *Nco*I and *Xba*I restriction sites between the promoter and the poly A signal. (B) Coding sequences for expression. PI, profilin I; MIIIt, C-terminal 256 residues of myosin-II tail; MII, full length myosin-II; MIIIm, full length myosin-II mutated at three phosphorylation sites to alanine; MIC, myosin-IC (b, basic region; g1, GPA1 domain; s, SH3 domain; and g2, GPA2 domain). Three phosphorylatable serines (S) in the tail piece of pUbGMIIIt and pUbGMII were replaced by three alanines (A) in pUbGMIIIm.



**Fig. 2.** Flow cytometry of a population of amoebas transfected with EGFP-fused profilin-I. M1, untransfected amoeba; M2, transfected cells useful for microscopy; M3, transfected cells with high fluorescence.

When expression was quantitated by flow cytometry, the EGFP fluorescence of individual cells varied in intensity by three orders of magnitude for any given expression vector (Fig. 2). Cells in the large peak with fluorescence  $< 50$  did not express EGFP. Cells in fraction M2 (fluorescence 90-1000) were alive and suitable for microscopy. The M2 fraction of cells expressing EGFP-profilin grew at the same rate as control

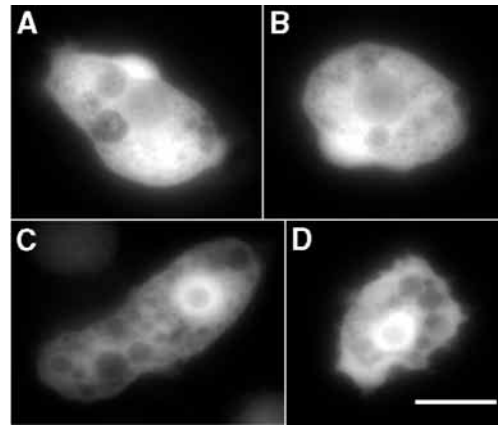


**Fig. 3.** Immunoblots to measure expression of profilin-I-EGFP in *Acanthamoeba*. Proteins were detected with antibodies to profilin, actin or GFP by chemiluminescence. T, amoeba transfected with pUbPG and selected from pools M2 and M3; C, control untransfected amoeba. Actin at 43 kDa served as the loading control for these samples. Endogenous profilins run at 13 kDa. The 42 kDa band that reacts with both anti-profilin and anti-GFP is the fusion protein. The band at 44 kDa that reacts with anti-GFP in both lanes T and C is a crossreactive amoeba protein. The minor 27 kDa band that reacted with anti-GFP but not anti-profilin is likely to be proteolytic fragment of EGFP.

cells, but over two weeks all of the cells lost their green fluorescence, probably owing to loss of the plasmid. The M3 fraction of cells with fluorescence  $>1000$  were round and immotile. The proportion of M3 cells was much larger in populations expressing EGFP-profilin than other constructs. These highly fluorescent cells rounded up before cell sorting, so their inactivity did not result from sorting.

Quantitation of expression levels showed that the highest levels achieved were in the micromolar range, below the concentrations of the endogenous native proteins, so the EGFP-fusion proteins were tracers in a population of these native proteins. We used cells expressing EGFP-profilin and EGFP-myosin-II tail fragment for quantitating expression, since they were expressed at higher levels than the larger constructs. We isolated transfected cells by cell sorting and compared the levels of expression of the fusion protein with the endogenous protein. Quantitative immunoblots (Fig. 3) showed that the cellular concentration of profilin-I-EGFP in the combined M2 and M3 fractions was 10% that of endogenous profilins (or 13% of the profilin-I), or about  $10 \mu\text{M}$  (that is  $10 \text{ nmoles/g}$  packed cells). Given the relative numbers of cells in M2 and M3 and their fluorescence (Fig. 2), M2 cells expressed  $0.3$  to  $3 \mu\text{M}$  EGFP-profilin-I, while M3 cells expressed  $3.5$  to  $35 \mu\text{M}$  EGFP-profilin-I. The average cellular concentration of EGFP-myosin-II tail fragment in the combined M2 and M3 fractions was about 20% that of endogenous myosin-II, or  $0.2 \mu\text{M}$ . These EGFP-myosin-II tail fragment samples had few cells in M3, so the cells used for microscopy expressed on average about  $0.1 \mu\text{M}$  fusion protein. In accordance with these measurements, cells expressing EGFP-profilin were moderately brighter by fluorescence microscopy than cells expressing EGFP-myosin-II tail. By both flow cytometry and fluorescence microscopy the expression of EGFP-myosin-II tail fragment was higher than the larger myosin fusion proteins. Thus, smaller constructs not only transfected a higher fraction of the cells but also were expressed at higher levels in these cells.

We placed EGFP at the N-termini of our myosin constructs, since N-terminal GFP-myosin-II complements null mutations in *Dictyostelium* (Moore et al., 1996; Zang and Spudich,



**Fig. 4.** Fluorescence micrographs of live amoebas expressing (A,B) profilin-I-EGFP and (C,D) EGFP. (A,B) Profilin-I-EGFP fills the cytoplasm and concentrates in pseudopods. (C,D) EGFP not only fills the cytoplasm but concentrates in nuclei except for the nucleolus. Bar,  $10 \mu\text{m}$ .

1998) and *S. pombe* (Bezanilla et al., 2000) and N-terminal GFP-myosin-I complements null mutations in *S. pombe* (Lee et al., 2000).

#### Distribution of EGFP and profilin-I-EGFP in live cells

Profilin-I-EGFP served as a control for our expressed myosin fusion proteins in live amoebas, since we could compare its behavior with that of rhodamine-labeled profilin-II introduced by syringe loading (Kaiser et al., 1999). The fluorescence of profilin-I-EGFP in live amoebas (Fig. 4A,B) was very similar to that of rhodamine-profilin-II and distinctly different from free EGFP. Profilin-I-EGFP filled the cytoplasm but was excluded from organelles including the nucleus. In contrast, free EGFP accumulated in the nucleus except for the nucleolus (Fig. 4C,D). Profilin-I-EGFP concentrated in pseudopods to a greater extent than free EGFP.

#### Distribution of EGFP-myosin-II in live cells

We studied myosin-II dynamics in live cells using two different EGFP-fusion proteins. The fluorescence was similar in cells expressing EGFP-full length myosin-II (Fig. 5A) and EGFP fused to the C-terminal 256 residues of the myosin-II tail (Fig. 5B,C). The EGFP-myosin-II tail construct produced better images, owing to the higher transfection efficiency and expression level (about 10% of the endogenous native myosin-II) compared with longer constructs. Our use of trace quantities of EGFP-myosin-II tail construct as a probe for myosin-II dynamics *in vivo* is justified by detailed studies of the assembly properties of the myosin-II tail. Truncations of the distal part of the myosin-II tail (Sinard et al., 1990) and experiments with monoclonal antibodies with carefully mapped epitopes (Rimm et al., 1990) showed that the C-terminal 100 residues are required for assembly. Biophysical analysis of purified myosin-II constructs has established that the C-terminal 256 residues of the myosin-II tail are sufficient to assemble an octamer equivalent to minifilaments of the full length protein (K. Turbedsky, Assembly of *Acanthamoeba* myosin-II, PhD thesis,

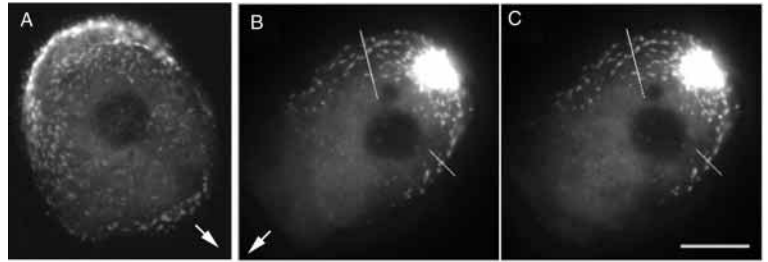
Johns Hopkins University, 2001; K. Turbedsky and T. D. Pollard, unpublished).

EGFP-full length myosin-II and EGFP-myosin-II tail were confined to the cytoplasm and concentrated in spots of two sizes. The large spots were asymmetric with dimensions of up to  $0.4 \times 0.8 \mu\text{m}$ , the same size as fluorescence images of thick filaments of purified myosin-II and myosin-II in fixed cells stained with a fluorescent monoclonal antibody (Yonemura and Pollard, 1992). Thick filaments are actually much smaller than their fluorescence images, being bipolar rods about 20 nm in diameter and 300 nm long (Sinard and Pollard, 1989). Smaller spots, presumed to be myosin-II minifilaments, were scattered throughout the cytoplasm but rarely at the very front of the cell. Small spots are readily visible as bright flecks when focusing through a cell with the microscope but difficult to reproduce in printed micrographs. Flattening transfected cells with an agar coverslip reduced superimposition (Yumura et al., 1984) and improved the resolution of both types of spots. Compression distorts the 3D architecture of the cell and is thought to stress *Dictyostelium* (Neujahr et al., 1997), but the behavior of EGFP-myosin-II was similar in most respects in 3D and flattened *Acanthamoeba*.

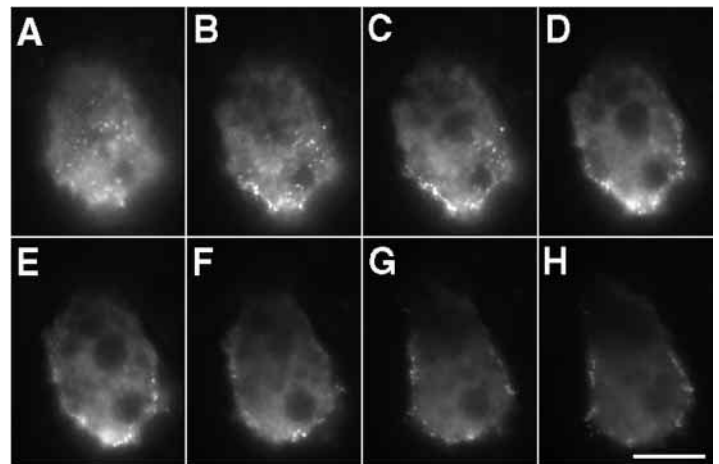
Large spots of EGFP-myosin-II concentrated at the trailing edge of cells (Figs 5-7) and spread out over the dorsal surface of unflattened cells (Fig. 6). Few large spots were present near the bottom surface. Small fluorescent spots of EGFP-myosin-II were more abundant in the internal cytoplasm than the cortex. In some transfected amoeba, large myosin-II spots aggregated at random positions including the posterior, anterior or mid-regions of the cytoplasm (Fig. 5B, Fig. 7A). These aggregates moved freely through the cytoplasm. Compression and/or the expression of the tail construct may have contributed to these artifactual accumulations of myosin-II.

Time-lapse records (Fig. 5B,C and see movie online; Fig. 7) revealed two types of myosin-II dynamics. The large spots of myosin-II underwent a cycle of assembly and disassembly on a time scale of tens of seconds. Large spots of myosin-II appeared de novo and grew into large fluorescent spots over about 60 seconds. In moving cells, most of the large spots disappeared near the front or along the sides. Thick filaments disappeared by losing their fluorescent intensity over about 60 seconds, most commonly near the rear of the cell (Fig. 7B). This represented disassembly rather than either (1) movement to another plane of focus (since the entire thickness of these flattened cells was in focus) or (2) photobleaching (since the fluorescence of other nearby filaments was stable).

Both types of EGFP-myosin-II spots moved continuously through the cytoplasm. Thick filaments moved from their sites of formation toward the rear of the cell. Some large spots increased in intensity as they moved backward. Those in Fig. 5B,C [see movie online (<http://jcs.biologists.org/supplemental>)] moved at about 25 nm/second, but their velocities varied within each cell. These movements resulted in the accumulation of bright spots at the rear where disassembly also took place. Small fluorescent spots in the deep cytoplasm moved forward. When an amoeba changed direction, the flow of spots reversed and



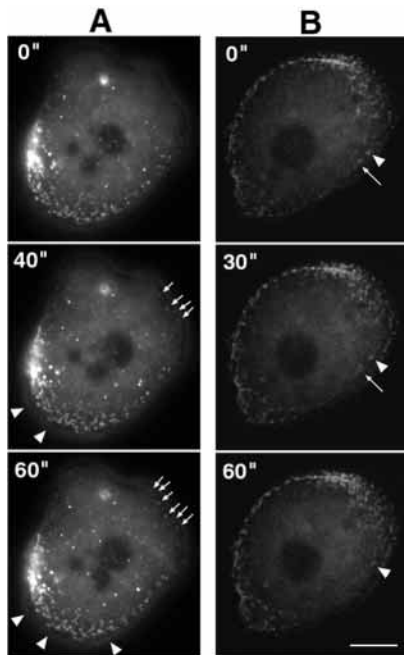
**Fig. 5.** Fluorescence micrographs of live amoebae expressing (A) EGFP-full length myosin-II or (B,C) EGFP-myosin-II tail fragment. The cells were flattened under an agar coverslip to reduce superimposition. The direction of movement is indicated with a white arrowhead. (A) Thick filaments labeled with EGFP-full-length-myosin-II concentrate at the trailing edge but are also found throughout the cytoplasm, including a few just behind the leading edge. (B,C) Fluorescence micrographs at 0 and 8 seconds. Intense fluorescent spots interpreted as thick filaments line up along the cell margin of the back half and move steadily backward toward a cluster at the posterior. Small fluorescent spots are interpreted as myosin-II minifilaments. Two white lines serve as positional reference marks. Bar, 10  $\mu\text{m}$ . See corresponding movie online (<http://jcs.biologists.org/supplemental>).



**Fig. 6.** Three dimensional distribution of myosin-II filaments in a live, unflattened amoeba expressing an EGFP-myosin-II tail fragment. Through a focus series of fluorescence micrographs: (A) section 1 from the dorsal surface; (B) section 3; (C) section 4; (D) section 6; (E) section 7; (F) section 10; (G) section 13; (H) section 16, ventral surface in contact with the slide. The cell was moving toward the upper left. Thick filaments are concentrated in the cortex over the dorsal surface especially the back half of the cell. Bar, 10  $\mu\text{m}$ .

thick filaments began to accumulate at the new trailing edge within seconds (Fig. 7A).

Phosphorylation of three serines in the tail piece of *Acanthamoeba* myosin-II inhibits the actin-activated ATPase in the heads (Kuznicki et al., 1983). We prepared a mutant EGFP-myosin-II, designed to be constitutively active through replacement of all three inhibitory heavy chain phosphorylation sites with alanine. Expression of this mutant myosin-II in cells containing wild-type myosin-II produced large and small fluorescent spots, similar to the spots of full length EGFP-myosin-II with wild-type phosphorylation sites, but the distribution of the spots was altered (Fig. 8). The large spots of constitutively active EGFP-myosin-II accumulated

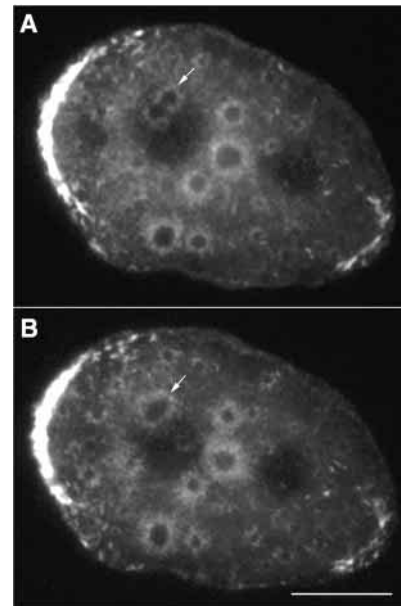


**Fig. 7.** Assembly and disassembly of myosin-II thick filaments. Two time series of fluorescence micrographs of live, flattened amoebas expressing EGFP-myosin-II tail fragment. Time points are given in seconds. (A) Appearance of new filaments. When this cell changed its direction of movement from upper right to lower left at about 20 seconds, new thick filaments (arrows) appeared within seconds at the new tail end as the new lamellipodium (arrowheads) emerged at lower left. The fluorescent intensity of these new filaments increased with time. (B) Disappearance of filaments. The filament marked with an arrow disappeared while a neighboring filament (arrowhead) moved toward the rear (upper right). Bar, 10  $\mu$ m.

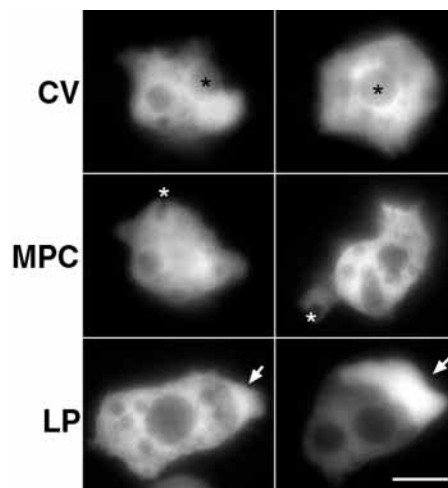
like a sunburst around vesicles, likely to be endocytic in origin. Some of these decorated vesicles fused. Wild-type EGFP-myosin-II never associated with such vesicles.

#### Distribution and dynamics of myosin-IC in live cells

EGFP-full length myosin-IC was distributed throughout the cytoplasm but concentrated in lamellipodia at the leading edge of moving cells and around contractile vacuoles and macropinocytic cups (Fig. 9). EGFP-myosin-IC accumulated transiently around contractile vacuoles only when they reached their maximal size, about 15 to 30 seconds before they discharged their contents, whereupon myosin-IC dispersed immediately (Fig. 10). Baines and Korn (Baines and Korn, 1995) reported that antibodies to myosin-IC stained the contractile vacuole in fixed cells throughout its cycle and that this myosin-IC was much more heavily phosphorylated at the time of contraction. The contrast in fixed cells may have been greater owing to some extraction of myosin-IC from the cytoplasm during fixation or permeabilization. The brief concentration around the contractile vacuole at the time of contraction might have been missed in fixed cells. We also found that EGFP-myosin-IC also accumulated around macropinocytic cups in live cells 3 to 5 seconds before their closure and then dispersed (Figs 9, 10). Myosin-IC was excluded from organelles and the nucleus.

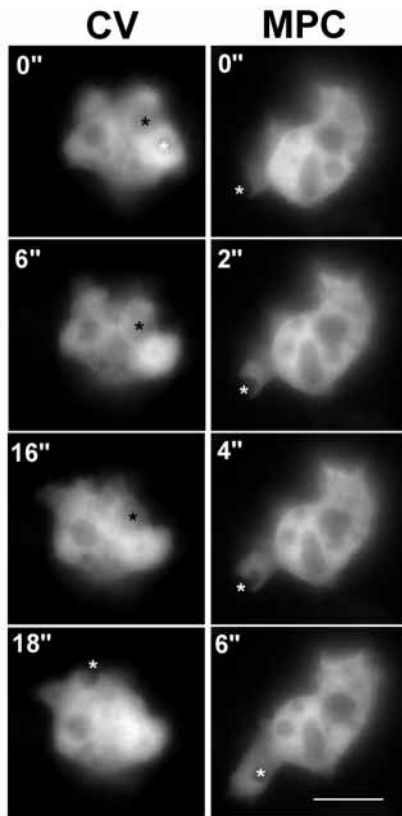


**Fig. 8.** Distribution of myosin-II filaments in a live, flattened amoeba expressing constitutively active EGFP-full length myosin-II with alanine substituted for serine at all three heavy chain phosphorylation sites. The leading edge is to the right. Fluorescence micrographs at (A) time zero and (B) 21 seconds. Fluorescence spots concentrated at the leading and trailing edge but also clustered around vesicles of various sizes. Two vesicles fused between these two time intervals (arrows). Bar, 10  $\mu$ m.



**Fig. 9.** Transient concentration of EGFP-myosin-IC around contractile vacuoles (CV, black asterisk), around macropinocytic cups (MPC, white asterisk) and in lamellipodia (LP, white arrow) in flattened amoeba expressing EGFP-myosin-IC. These fluorescence micrographs were taken 1 second before contraction of the contractile vacuoles and closure of the macropinocytic cups. Bar, 10  $\mu$ m.

Expression of EGFP fused to N- or C-terminal truncation constructs showed that both the myosin head and the SH3 domain in the tail are required for myosin-IC to localize to contractile vacuoles and macropinocytic cups (Fig. 11). Over 30 cells were observed for each construct. The EGFP construct lacking just the C-terminal GPA domain (MICs) localized



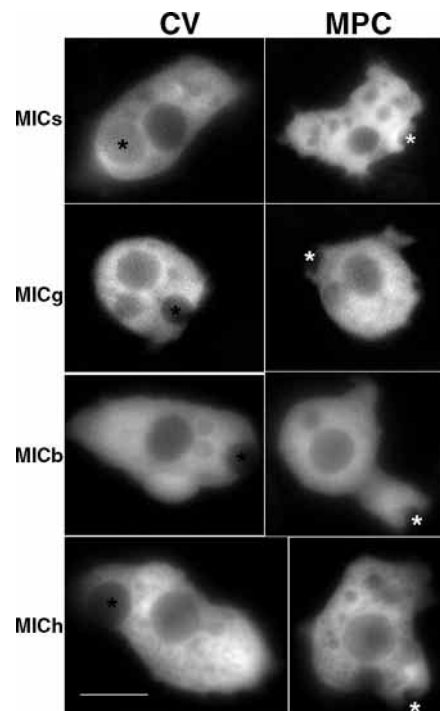
**Fig. 10.** Time sequences of fluorescence micrographs of two flattened cells expressing EGFP-myosin-IC. Fluorescence concentrates around a contractile vacuole (CV, black asterisk) just prior to contraction and macropinocytic cups (white asterisk) just before closure. The series of white asterisks trace the ingestion of medium by a macropinocytic cup (MPC). Time is in seconds. Bar, 10  $\mu$ m.

much like the full length construct in the cytoplasm and around contractile vacuoles and macropinocytic cups. EGFP-myosin-IC constructs lacking the SH3 (MICg) or GPA and SH3 (MICb) domains of the tail were distributed throughout the cytoplasm and did not concentrate around contractile vacuoles or macropinocytic cups. Constructs consisting of head (MICH) or tail alone (not shown) did not localize around contractile vacuoles or macropinocytic cups. However, all truncated myosin-IC constructs, even those lacking the SH3 domain or head, did concentrate in lamellipodia. Contractile vacuoles filled and contracted normally in cells expressing each of the myosin-IC constructs.

## Discussion

### Assembly and localization of myosin-II

As in fixed cells stained with fluorescent antibodies to myosin-II (Yonemura and Pollard, 1992), EGFP-myosin-II concentrated in particles of two sizes in the cytoplasm of *Acanthamoeba*. Yonemura and Pollard concluded that the small fluorescent spots are mini-filaments and the large spots are thick filaments, based on comparison of cells and filaments of purified myosin-II stained with directly-labeled fluorescent antibodies (Yonemura and Pollard, 1992). Thick filaments form by lateral association of mini-filaments, each consisting of



**Fig. 11.** Behavior of EGFP-myosin-IC truncation mutants. Fluorescence micrographs of flattened cells expressing EGFP fusions with truncated myosin-IC constructs taken 1 second before contraction of the contractile vacuoles (CV, black asterisks) and closure of the macropinocytic cups (MPC, white asterisks). MICs lacks the C-terminal GPA2 region (residues I1051 to M1186). EGFP-MICg lacks the C-terminal SH3 and GPA2 region (residues P996 to M1186). EGFP-MICb lacks the C-terminal GPA and SH3 domains (residues L940 to M1186). EGFP-MICH lacks the entire tail (residues N720 to M1186). Constructs with the head, basic, GPA1 and SH3 domains concentrated around the contractile vacuoles and macropinocytic cups. Constructs lacking the SH3 domain distributed diffusely in the cytoplasm. Bar, 10  $\mu$ m.

eight myosin-II molecules (Sinard et al., 1989). Assembly of virtually all of the myosin-II into such filaments, as observed here, is expected from the highly favorable equilibrium constants for assembly [ $K_d=20$  nM (Sinard et al., 1990)] and the cytoplasmic concentration of myosin-II ( $\sim 1$   $\mu$ M).

Expression of EGFP-myosin-II allowed us to observe a cycle of assembly and disassembly of thick filaments in *Acanthamoeba* for the first time. Thick filaments concentrated in the dorsal cortex and along the lateral edge in the back half of the cells, while minifilaments were dispersed more uniformly throughout the cytoplasm. Thick filaments form and grow in size over a period of seconds. New thick filaments usually assemble behind the leading edge and then move dorsally and laterally by cortical flow toward the rear of the cell where they disassemble. Given the stability of minifilaments under physiological conditions (Sinard and Pollard, 1989), we suggest that thick filaments disassemble into minifilaments, which then move forward by bulk cytoplasmic flow for reassembly in more anterior regions of the cell. However, minifilaments are too small to follow individually with enough confidence to be certain about their stability. Millimolar concentrations of divalent cations and acid pH favor the lateral

association of pure myosin-II mini-filaments into thick filaments (Sinard et al., 1990). In vivo, it seems more likely that yet to be identified accessory proteins rather than ionic conditions regulate thick filament assembly.

Mutation of three phosphorylatable serines in the non-helical tail piece had a greater impact on the localization of myosin-II than removing the head and proximal tail. EGFP fused to the C-terminal 256 residues of myosin-II behaved just like full length myosin-II, most likely owing to co-assembly with the more abundant wild-type myosin-II. This tail construct include all of the elements required to assemble octameric minifilaments in vitro (K. Turbedsky, PhD thesis, Johns Hopkins University, 2001) and may suffice to specify many functions. By contrast, expression of a low mole fraction of unphosphorylatable myosin-II caused the filaments to cluster around vesicles more than wild-type myosin-II. Three serines in the tail piece are heavily phosphorylated in vivo (Collins and Korn, 1981). Although phosphorylation of the tail piece serines has no effect on assembly of mini-filaments or thick filaments (Sinard and Pollard, 1989; Redowicz et al., 1994), it does inhibit binding to actin filaments and the actin-activated ATPase of the heads (Cote et al., 1981; Collins et al., 1982a; Collins et al., 1982b; Ganguly et al., 1992). Dephosphorylation allows actin filaments to activate the ATPase activity, and so the triple alanine mutant is expected to be constitutively active. Constitutively active myosin may bind to actin filaments associated with cytoplasmic vesicles.

#### Comparison with the behavior of myosin-II in other cells

Although the best characterized cytoplasmic myosin-II filaments (from vertebrate cultured cells, *Dictyostelium* and *Acanthamoeba*) appear to differ in the mechanisms regulating their assembly and localization, a few general themes are now apparent. Differences include regulation of assembly by heavy chain phosphorylation in *Dictyostelium* (Kolman et al., 1996) but not the other systems, regulation of assembly by phosphorylation of the regulatory light chain in vertebrate cells (Kolega and Kumar, 1999) but not the other systems, and the association of myosin-II filaments with stress fibers in slowly moving vertebrate cells (Kolega and Taylor, 1993) but not in rapidly moving cells. Nevertheless, in all three systems myosin-II filaments concentrate at the rear of motile cells (Moores et al., 1996; Clow and McNally, 1999), where they generate retraction forces. The following paragraphs discuss two other features that these systems have in common.

#### Lateral clustering of minifilaments

*Acanthamoeba* and vertebrate myosin-II form stable minifilaments that associate laterally into larger assemblies both in vitro (Sinard et al., 1989; Niederman and Pollard, 1975) and in cells (Yonemura and Pollard, 1992; Svitkina et al., 1997; current report). The function of these larger assemblies has not been established experimentally, but is probably related to the greater force produced by bringing together many heads in one physical unit. The assembly mechanism of *Dictyostelium* myosin-II is less well understood, but the appearance of large fluorescent spots in cells stimulated with cAMP (Yumura et al., 1984) may also represent assembly of thick filaments from mini-filaments or smaller oligomers.

#### Rapid recycling of myosin filaments

Migrating human fibroblasts (Kolega and Taylor, 1993; DeBiasio et al., 1996) and fish epidermal keratocytes (Svitkina et al., 1997) assemble myosin-II filaments in deep parts of lamellipodia. In keratocytes discrete clusters of bipolar minifilaments increase in size and density towards the perinuclear area and concentrate in transverse actin filament bundles. These myosin clusters remain stationary with respect to the substratum in locomoting cells, but they exhibit retrograde flow in cells tethered in epithelioid colonies. The myosin-II assembly cycle is similar in *Acanthamoeba*. Thick filaments form anteriorly and move toward the rear where they disassemble. Thick filaments appear to assemble from and disassemble into mini-filaments, but the regulatory mechanisms have yet to be identified. *Dictyostelium* myosin-II filaments also turn over rapidly with a half-life of 7 seconds in the cleavage furrow (Yumura, 2001). Reversible phosphorylation of the *Dictyostelium* myosin-II heavy chain regulates assembly in vitro (Kuczmariski and Spudich, 1980) and in vivo (Kolman et al., 1996; Yumura, 2001). PAK (p21 activated kinase) also influences the assembly and localization of *Dictyostelium* myosin-II (Chang and Firtel, 1999).

#### Structural requirements for transient association of myosin-IC with contractile vacuoles and macropinocytic cups

The bulk of EGFP-myosin-IC spreads uniformly throughout the cytoplasm, but it concentrates for a few seconds around contractile vacuoles prior to their discharge and on the cytoplasmic surface of the plasma membrane participating in macropinocytosis prior to closure. In both cases myosin-IC dissipates quickly after membrane fusion. More EGFP-myosin-IC appeared in the cytoplasm between the organelles of live cells than in fixed cells (Baines and Korn, 1990; Baines et al., 1995). Although the role of myosin-IC in contractile vacuole function has yet to be defined, it is the only myosin-I isoform detected around contractile vacuoles with antibody staining (Baines and Korn, 1990; Baines et al., 1995). Microinjection of antibodies to myosin-IC inhibited contractile vacuole function, while injection of antibodies to myosin-IA or -IB did not (Doberstein et al., 1993). In parallel work we labeled a monoclonal antibody specific for myosin-IA and -IC with the fluorescent dye Cy3 and introduced it into live cells by syringe loading (Ostap et al., 2003). This non-inhibitory fluorescent antibody associated transiently with macropinocytosis cups and to a lesser extent with contractile vacuoles. Comparison with EGFP-myosin-IC in this study suggests that myosin-IA is more enriched around macropinocytosis cups and myosin-IC is enriched around contractile vacuoles.

Five different truncation mutants revealed that localization of myosin-IC to contractile vacuoles and macropinocytosis cups requires both the heads and the SH3 domain in the tail, while the second TH2 domain at the C-terminus is dispensable. In *Acanthamoeba* myosin-I heads provide ATP-sensitive actin binding, the TH1 domains interact with both acidic lipids (Doberstein and Pollard, 1992) and actin filaments (Lee et al., 1999), TH2 domains reinforce actin filament binding (Lynch et al., 1986; Lee et al., 1999) and SH3 domains associate with Acan125 (Xu et al., 1995; Xu et al., 1997; Lee et al., 1999),

the founding member of the CARMIL family of adapter proteins. The *Dictyostelium* CARMIL protein interacts with both Arp2/3 complex and heterodimeric capping protein (Jung et al., 2001). Since the SH3 domains of both myosin-IA and -IC bind Acan125 with high affinity (Lee et al., 1999; Zot et al., 2000), other determinants must contribute to the distinct localizations of these two myosin-I isoforms (Baines et al., 1995).

The available evidence suggests that the head and tail domains required for biological function vary among myosin-IIs from various species. Vertebrate myo1b requires both heads and tail domains for localization to membrane ruffles (Tang and Ostap, 2001). In *Dictyostelium*, expression of a mutant myoB lacking the SH3 domain failed to rescue the defects in myoA<sup>-</sup>/myoB<sup>-</sup> double mutants (Novak and Titus, 1998), although *Dictyostelium* myoB lacking the SH3 domain localized normally. In *Aspergillus*, deletion of the SH3 domain of MYOA has no effect on cell growth, morphology, secretion or endocytosis (Oshero et al., 1998), while the SH3 domain of budding yeast Myo5p is required for full function and subcellular targeting (Anderson et al., 1998). The SH3 domains of budding yeast myosin-IIs participate in actin assembly by binding the actin-associated protein verprolin, the fungal homolog of WASp-interacting protein, WIP (Roberson et al., 1997; Lechler et al., 2000; Evangelista et al., 2000). By contrast, only the head and basic region (TH1) of the single fission yeast myosin-I, Myo1p, are required for cortical localization and to complement the defects of myo1 deletion (Lee et al., 2000). However, the SH3 domain of Myo1p is required for full function in the absence of the WASp homolog Wsp1p. Since a consistent picture of the relation of tail domains to function has yet to emerge, these myosin-IIs may genuinely differ from each other.

Insights from the fungal systems and the discovery of the CARMIL family of adapter proteins emphasize that myosin-I is part of a complex network of interacting proteins that regulate actin assembly. Further work is required to learn how the motile activities and actin assembly activities of the various myosin-I filaments contribute to their biological functions. Such efforts to relate structure and function will have to take into account all of the components of these systems.

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